Human Blood Groups and Antibodies

Phyllis Patricia Moores

Nat. Dip. Med. Lab. Tech (S.A.) B.S.c. (Bristol)

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Frontispiece

SOUTH AFRICANS

ABSTRACT

The following blood group phenotypes and antigens were studied: A_{bantu}, A_x, A_Y, B_m, B_m-like, B_3-like, "Bombay" O_h Le(a+b-), "Bombay" O_h Le(a-b-), para-Bombay, Mi(a+), Vw+, S-s-U-, Dantu, Gerbich-, P_1H, STEM+, Rh:-34, Rh_{null}, Le(a-b-c-d-), McC(e+) and Wd(a+) and a new form of polyagglutination associated with haemoglobin M - type Hyde Park. The effect of inheriting a *y*, *D*--, *Dc*- or R^{1Lisa} haplotype was also investigated.

The following blood group antibodies were studied: anti-N in a person with type MN red cells, anti-hr^s, anti-Rh34, anti-Js^b and anti-T. Type M red cells were confirmed to absorb anti-N and type N red cells not to absorb anti-M.

A new technique was described for separating the two red cell populations in twin chimeras. Three XX/XX female dispermic chimeras with blood of two genetic types, two with patchy skin pigmentation, were identified. Reduced I and enhanced i antigen expression helped confirm a case of congenital dyserythropoietic anaemia type II. Oval red cells accompanying an *r* (*dce*) haplotype were found, and anti-Tj^a-like haemolysins were not detected in women about to abort.

Aspects of haemolytic disease of the newborn due to ABO and Rh antibodies were discussed. Two new tests in which 2-mercapto-ethanol was used to distinguish between IgG (7S) and IgM (19S) immunoglobulins were described. Blood group phenotype and gene frequency studies were made in Black, White, Indian and Coloured blood donors and the results were presented in 32 tables. Thirty monoclonal anti-A and 96 monoclonal antibodies for antigens in the ABO, MNSs, Rh, Lutheran, Kell, Lewis and Kidd systems and for other antigens were investigated for their activity and specificity.

PREFACE AND ACKNOWLEDGEMENTS

This thesis includes all my original and co-authored publications to date, some original but still unpublished studies from my M.Sc. (1976) and Ph.D. (1981) theses, and thirty-two recently formulated tables containing the results of my blood group phenotype and gene frequency studies in blood donors of the Natal Blood Transfusion Service. The frequencies in the Black donors are from my M.Sc. thesis, those in the White donors from the same thesis, in which they were included as the control group, those in the Indian donors from my Ph.D. thesis and those in the Coloured donors from a published paper and another accepted for publication but that has not yet appeared in print. None of this work has been submitted in any form for any degree or diploma to any University other than the University of Natal. All the investigations since 1961 were made at the Natal Blood Transfusion Service and Natal Institute of Immunology, where I am employed as a full-time staff member.

The chapters begin with a list of my publications relevant to each title. This is followed by a short introduction in which the earlier related work is briefly reviewed. Commentaries on the the papers are then furnished, in the form of summaries. The thesis concludes with a brief résumé of the rare and interesting blood groups and conditions identified in southern Africa.

Born in Bournemouth, England, in 1927, I travelled extensively around Africa as a child with my parents. My father was a wireless engineer on foreign service for Cable and Wireless, a British telecommunication company. After having matriculated in Salisbury (now Harare), Southern Rhodesia (now Zimbabwe) in 1944 and having studied pharmacy for two years at the British Naval and British Army hospitals in Alexandria, Egypt, I was taken to England and enrolled as an undergraduate at Bristol University. In 1949, the University awarded me the degree of Batchelor of Science. Later in the same year, I rejoined my parents, who by now had settled in Durban, and was employed by the Natal Provincial Pathological Laboratory Service as its

first post-graduate medical technology student. In 1951, the South African Medical and Dental Council awarded me its National Diploma in Medical Laboratory Technology (Clinical Pathology), and I was registered as Medical Technologist No. 27. After two years as Technologist in Charge, Rhesus Laboratory, I entered the Natal Blood Transfusion Service (NBTS) as Technologist in Charge, Crossmatching and Antenatal Laboratories, Addington Hospital. Early in 1955, following my parents' divorce, I returned to England. During the first six months in London, Professor J.V. Dacie employed me as a senior medical technologist in his Special Haematological Investigations laboratory at the Post-graduate School of Medicine, Hammersmith Hospital. Dr R.R. Race and Dr R. Sanger then kindly asked me to join them in the Blood Group Research Unit at the Lister Institute in Chelsea Bridge Road. Two very happy and productive years were spent with them as their senior research assistant; this was also a most exciting time to be engaged in blood group serology. It is my earnest hope that my subsequent work has been worthy of this excellent training and experience. The next four years were passed in Salisbury (Harare) as a medical technologist in the private pathological laboratory of Dr G.V. Blaine. Among general haematological, parasitological, biochemical and other studies, I was responsible for all the antenatal investigations and for grouping and crossmatching the blood donations for the local Blood Transfusion Service. In 1961, my mother having become terminally ill, I returned once more to Durban, where I was fortunate enough to be asked by Dr B.G. Grobbelaar, then Medical Director of the Natal Blood Transfusion Service, to found and develop a Special Investigations Laboratory for advanced blood group and antibody studies. In 1976, the University of Natal awarded me the degree of Master of Science for my thesis entitled "The Blood Groups of the Natal Negro People", and I was promoted to the position of Scientific Officer. In 1981, the University of Natal awarded me the degree of Doctor of Philosophy for my thesis entitled "The Blood Groups of the Natal Indian People". By now I had been employed as Head of the Red Cell Serology Reference Laboratory at the NBTS for some years. Late in 1987, I was transferred to

the newly-created Immuno-Haematology Reference Laboratory at the Natal Institute of Immunology (Research Division of the NBTS); and this is my position at present.

Since 1965, papers and/or posters have been presented at all except one of the National Blood Transfusion Congresses and at other congresses and meetings in South Africa. Papers and/or posters were also presented in 1972, 1975, 1978, 1982, 1984, 1986, 1987 and 1990 at International Blood Transfusion and other Congresses and meetings overseas. In 1988 and 1990, I was invited to attend and took part in the preliminary investigations for the First and Second International Workshops on Monoclonal Antibodies, which were held in Paris, France, and in Lund, Sweden, respectively.

After 30 years of very happy and productive work in Natal, during which many opportunities arose to make interesting and meaningful blood group studies, I can truthfully say that my career has been a most satisfying and scientifically rewarding one. Natal, with its four main populations: Black, White, Indian and Coloured, has been, and still is, a splendid place in which to have engaged in these investigations. The all-absorbing interest which the groups raised, however, was not the only reason for studying them; they also had real practical importance. When patients with rare groups and antibodies needed blood urgently, it was essential to find donors with the same rare groups quickly. Through knowing the donors with rare blood groups personally and where they might be found, these loyal men and women, most of whom subsequently became members of the Rare Donor File, frequently came to the rescue by providing donations of their compatible blood. Their co-operation has been very much appreciated.

I would like to thank Dr J.C. Thomas, late Medical Director, Natal Provincial Pathological Laboratory Service, for having provided me with my first opportunity to group blood when he appointed me Head of this Service's Rhesus Laboratory in 1951. I am also deeply indebted, as already described, to the late Dr R.R. Race and to Dr R. Sanger, Blood Group Research Unit,

London, who inspired in me a deep and long-lasting love for blood group research. Without them, and their continued help and advice, I would not have achieved so much.

To Dr P. Brain, recently retired Medical Director, Natal Blood Transfusion Service, I owe immense thanks for his sustained encouragement and advice: no-one could have a better friend. Dr G.H. Vos, Natal Institute of Immunology, who for some years was almost the only person with whom I could converse in the intricate language of blood group serology, was a respected comrade until he retired in 1988. Mr L.V. Milner, my immediate superior and colleague, stoically and patiently endured my abundant joys, sorrows and tears. Mrs Elizabeth Smart, who ably assisted me from 1976 to 1987, is still a most loyal and devoted colleague. I thank her, most sincerely, for having allowing me to make good use of her technical skill. To the multitude of staff members, blood donors and patients who so willing supplied me with samples of their blood and saliva on numerous occasions, my many co-authors, colleagues and friends in other Blood Transfusion Services and laboratories throughout South Africa and the world, I give my most heartfelt thanks. This has been a lifetime of incredible interest and excitement, and it will be a wrench to have to leave it so soon upon retirement.

I would like to thank Professor C. Fernandez-Costa, Medical Director and Chief Executive of the Natal Blood Transfusion Service, the Executive Committee of this Service and Dr J. Conradie, Director of the Natal Institute of Immunology, for having kindly given me permission to prepare and present this thesis. I am also deeply grateful to Miss C. Ouwerkerk, Miss T. Barham, Mrs F. Green, Dr M. Bubb and Mr D. Stubbings, and to Mr S. Gengaya and his staff in the printing department, for their part in its production.

Lastly, but by no means least, I must thank the good Lord sincerely for having provided me with the necessary health, strength, skill and persistance to investigate the fantastic array of interesting blood samples that He constantly showered

upon me. At times, the samples arrived so fast that, until now, few if any moments remained to publish papers about them. It seemed more appropriate to identify each problem quickly, find compatible blood and provide the sender with a reasonably detailed report.

Durban, December 1991. Phyllis Patricia Moores

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TABLES

Position of South Africa on the continent of Africa

MAP OF SOUTHERN AFRICA

CHAPTER I

THE ABO SYSTEM

I.1 PAPERS AND STUDIES

A gene, *y*, modifying the blood group antigen A.
Paper 5 by Weiner, Lewis, Moores, Sanger and Race.

The A subgroups of the ABO blood group system.
Paper 11 by Moores.

Subgroups of A and AB in Natal Negroes.
Unpublished study 1, M.Sc. thesis, p41-57.

Weak group A variant phenotypes in the Natal Indians.
Unpublished study 2, Ph.D. thesis, p41-49.

Weak group B variant phenotypes in the Natal Indians.
Paper 25 by Moores; and
Unpublished study 3, Ph.D. thesis, p50-72.

The first "Bombay" O_h person in South Africa.
Unpublished study 4, Ph.D. thesis, p82-89.

Red cell and serum studies in the first family to have been recorded in which both O_h Le(a+b-) and O_h Le(a-b-) siblings were represented.
Papers 26a and 26b, by Moores; and
Unpublished study 5, Ph.D. thesis, p90-102.

Haemagglutination inhibition studies for the evaluation of blood group antigens in ethanol soluble substances (ESS) obtained from human, baboon and Vervet monkey red blood cells.
Paper 32 by Vos, Moores, Downing and Mohideen.

Haemagglutination inhibition studies of water soluble blood group substances recovered from the erythrocytes of classical "Bombay" O_h subjects.
Paper 33 by Vos and Moores.

Some observations on "Bombay" bloods, with comments on evidence for the existence of two different O_h phenotypes.
Paper 27 by Moores, Issitt, Pavone and McKeever.

Serological studies of anti-A+B+H allo-antibodies in the sera of three Natal "Bombay" O_h Indians.
Unpublished study 6, Ph.D. thesis, p129-140.

I.2 INTRODUCTION

The human blood groups A, B and O were discovered in the year 1900 and 1901 by Landsteiner. In 1910, von Dungern and Hirszfeld confirmed that the groups were inherited characters, and in 1924 Bernstein explained the method by which they were inherited. Hirszfeld and Hirszfeld showed in 1919 that the distribution of the groups differed among the world's populations. Subgroups of group A were first recognised in 1930 by Thomsen, Friedenreich and Worsaae; and by 1936 both Fischer and Hahn and Friedenreich had extended their number significantly. Subgroups of group B were first recognised in 1955 by Mäkelä and Mäkelä. The "Bombay" or O_h phenotype, in which the red cells type as O but the plasma contains antibodies active against A, B and O red cells, was identified in 1952 by Bhende, Deshpande, Bhatia, Sanger, Race, Morgan and Watkins. Para-Bombay phenotypes were reported for the first time in 1961 by Levine, Uhlíř and White.

I.3 COMMENTARY

I.3.1 Attainments in London, 1955 to 1957

The *Y/y* genes were discovered during the time that the author worked as research assistant to Dr R.R. Race and Dr Ruth Sanger at the Blood Group Research Unit, Lister Institute, London. In paper 5, homozygous *y/y* genes were described which inhibited A^1 gene expression and depressed the secretion of A substance in the saliva. The expression of the *B* and *H* genes was not affected. The red cells of the propositi were agglutinated neither by anti-A nor anti-A,B but they combined with anti-A and gave it up readily in eluates. Family studies suggested that the locus for *Yy* was not closely linked to the *ABO* locus.

I.3.2 Attainments in Durban, 1961 to 1991

1.3.2.1 Subgroups of A

On recommencing work in Durban, South Africa, in 1961, the author found that the subgroups of A weaker than A_2 were all being described locally merely as "weak A", despite their known

considerable frequency in the Black population [Shapiro, 1951]. As anti-A_1 was often present in the plasma, the Natal Blood Transfusion Service had for some time employed group A_2 instead of group A_1 red cells for ABO reverse-typing tests. Negative results with the A_2 red cells ensured that the donors with "weak A" red cells were not mistyped as O. In 1966, Brain classified the subgroups of A in the Blacks as A_1, A_{int}, A_2 and a previously undescribed weak form a characteristic of which was that the red cells were agglutinated by anti-A in an A_3-like pattern. The weak form was given the name A_{bantu}.

For the author's own information and in response to the considerable interest of local technologists in the subtypes of A, paper 11 recorded the characteristics of all the subgroups known in 1967 in parallel in a single large table. The modifications associated with leukaemia, chimerism and inhibitor genes were included. The paper emphasised the value of classifying the subgroups under the two general headings, A_x and A_m, as suggested by Race and Sanger [1962, p28-30]. It also gave practical hints on subgroup A identification.

During the years which followed, the frequencies of the subgroups A_1 and A_{bantu} in the Natal Blacks were estimated and recorded [study 1]. Their A_1 frequency was one of the highest in Africa. Their A_{bantu} frequency agreed with the 4% frequency recorded in 1966 by Brain. In the group AB Natal Blacks, H antigen was surprisingly present or absent irrespective of the A subgroup phenotype.

Two Natal Indians with subgroup A_x red cells were described in study 2. Their phenotype was readily distinguished from A_{bantu}. Two other Natal Indians with A_m-like red cells were also recorded, but their family studies were uninformative. When their red cells were exposed to anti-A, these antibodies were difficult to elute, and the saliva of one propositus contained only a minimal amount of A substance. These findings were characteristic of the rare subgroup-A phenotype A_y.

I.3.2.2 Subgroups of B

No previous records of weak B phenotypes in South Africa were found. Using as a guide the subgroup B classification suggested by Race and Sanger [1975, p19-20], two Natal Indian families with Category 2 or B_m red cells, one containing eleven affected members, were reported [paper 25 and study 3]. Four other Natal Indians with Category 2-like or B_m-like red cells and two with Category 3-like or B_3-like red cells were also found. As no B subgroups were identified in the Natal Black or the Natal White populations, these phenotypes were evidently inherited Indian characteristics. The B_3-like were not a mixture of B and O red cells as the B antigen was weaker than normal.

I.3.2.3 The "Bombay" O_h phenotype

The first South African with "Bombay" O_h red cells was identified in Durban in 1964 [study 4]. The proposita was an elderly Indian woman whose home language was Telegu. She was suffering from carcinoma of the cervix and, despite extensive testing, no compatible blood had been found for her. Her case was presented at the South African Blood Transfusion Congress in 1965. The woman was a non-secretor of ABH substances and her red cells were Lewis phenotype Le(a+b-). A family study showed that she had either A^1/O, B/O or A^1/B genes. The antibodies in her plasma reacted with A_1, B and O red cells to titre 32 by saline, 256 by enzyme and 512 by indirect antiglobulin technique and were not a simple mixture of anti-A, anti-B and anti-H. Twenty-three further Natal Indians and members of other population groups with the same phenotype were subsequently identified. All were shown to be the "Classical" type, in which no A, B or H antigen is demonstrable on the red cells and the antibodies agglutinate A, B and O cells all to approximately equal titres.

A Natal Indian family containing both O_h Le(a+b-) and O_h Le(a-b-) siblings was recorded in 1969 [paper 26]. Race and Sanger noticed [1975, p23, quoted by kind permission of

Blackwell Scientific Publications Ltd, Osney Mead, Oxford, England.] that the family confirmed the independent segregation of the *h* and *Lewis* genes. In subsequent titrations with Bombay antibodies and *Ulex europaeus* anti-H lectin study 5, the red cells of the family members with *H/h* genes were found to carry as much H antigen as those of the members with *H/H* genes and the group O controls. Dr Winifred M. Watkins kindly assayed the family A transferase activity in the family. The parents had significantly less α-2-L-fucosyltransferase than the four group O controls. The single child tested, who might have *H/H* or *H/h* genes, unfortunately gave intermediate results.

In another study, ethanol-soluble substances prepared from a sample of O_h red cells were found to inhibit the agglutination of O red cells by the donor's own antibodies [paper 32]. This suggested that O_h red cells had "hidden" H antigen, perhaps in the form of cryptantigen. The amount of H substance differed little from that recovered from group A_2 red cells. Variable quantities of water soluble H substance were also obtained by the same technique from the red cells of seven different O_h Indians [paper 33]. Type IgG was more sensitive than type IgM anti-H in detecting it. The obligate *H/h* parents in the family in paper 26 and study 5 had approximately the same amount of H antigen as the group O controls; the secretor parent had more than the non-secretor parent. Moreover, the water soluble substances had Lewis antigen activity. Those recovered from O_h Le(a+b-) red cells inhibited anti-Le^a to approximately the same titre as the substances from ordinary O Le(a+b-) red cells. The substances from ordinary O Le(a-b-) red cells inhibited anti-Le^a less strongly or not at all.

Serological studies were made with the antibodies of 19 Natal "Bombay" O_h Indians and enzyme-treated O_h red cells. This more stringent technique, however, failed to detect any evidence that the cells had H antigen [study 5]. Although in some cases the cells appeared agglutinated weakly, this was more likely to be aggregation caused by the enzyme-treatment. Such treatment is known to partially overcome the natural forces of repulsion between red cells.

An attempt was made to determine whether the positive results recorded by others with the eluates recovered from O_h red cells after exposing these cells to anti-A, anti-B and anti-H reagents were due to increased I antigen, unsuspected anti-I in the reagents and the Matuhasi-Ogata phenomenon [paper 27]. Samples of known $O^A{}_h$, $O^B{}_h$ and $O^o{}_h$ cells were mixed separately with anti-A, anti-B and anti-H known to contain anti-I. Eluates were made, and the results were recorded "blind". Neither anti-A, nor anti-B nor anti-H was detected. The findings suggested that at least two forms of O_h red cells existed, one representing total and the other marked but not total suppression of H, A and B antigens. The latter form partially inhibited production of the corresponding antibody in the serum.

Multiple absorption-elutions were made with the antibodies of Natal O_h Indians and A_1, B and O cells [study 6]. The results confirmed that their anti-H, rather than their anti-A and anti-B, was serologically their most significant antibody. The eluates from the A_1 cells were found to contain stronger anti-H than those from the B cells, and the eluates from the B cells to contain stronger anti-H than those from the O cells. The anti-H often present in the sera of group A_1 persons, and *Ulex europaeus* anti-H lectin, characteristically agglutinate or are absorbed by red cells from strongly to weakly in the order O - B - A_1. The group O and group A_2 babies of O_h mothers were also at greater risk than their group A_1, group B and group AB babies from haemolytic disease of the newborn due to Bombay antibodies. The study unexpectedly revealed a further way of using Bombay antibodies to prepare anti-H in pure form.

I.3.2.4 Para-Bombay phenotypes

Three Natal Indians with para-Bombay red cells of types $O^A{}_{Hm}$, $O^B{}_{Hm}$ and $O^o{}_{Hm}$ respectively were identified in study 7. No previous records of this phenotype in South Africa were found. Family studies were possible only in the two latter cases. All three Indians were secretors of ABH substances and had weak, type IgM, cold antibodies in their sera. Their red cells

reacted weakly with Bombay antibodies but gave negative results with *Ulex europaeus* anti-H lectin. Solomon, Waggoner and Leyshon [1965] suggested that para-Bombay red cells were due to the inheritance of a recessive suppressor gene situated at a locus *Zz*. In this study, the unexpectedly enhanced H antigen detected in a sibling of the O^{B}_{Hm} proposita was explained by postulating the existence of a mutant Z^{1} gene.

Paper 5

VOX SANGUINIS

Journal of Blood Transfusion and Immunohaematology – Journal de Transfusion Sanguine et d'Immunohématologie – Zeitschrift für Bluttransfusion und Immunohämatologie

Bulletin de la Société Internationale de Transfusion Sanguine

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A Gene, y, Modifying the Blood Group Antigen A

W. WEINER, H. B. M. LEWIS, Phyllis MOORES, Ruth SANGER and R. R. RACE

National Blood Transfusion Service, Birmingham
Medical Research Council Blood Group Research Unit, The Lister Institute, London

Two loci are known whose genes influence the expression of the *ABO* genes: the secretor locus, *Ss*, of *Schiff* and *Sasaki* [6] (1932) and the *Xx* locus of *Levine*, *Robinson*, *Celano*, *Briggs* and *Falkinburg* [5] (1955) which is responsible for the rare "Bombay" blood group.

The present paper gives evidence of the existence of a third such locus, *Yy*. The genotype *yy* modifies the development of the A antigen in the red cells and, to a much less extent, in the saliva. The details of the phenotype depend on the particular combination of the A_1A_2BO genes with *yy*.

It is probable that the phenotype A_x of *Gammelgaard* and the phenotype A_m of *Wiener* and *Gordon* are due to the *yy* genes.

In 1942 *Gammelgaard* [3] published a very fine M.D. thesis on the subject of weak A groups; the thesis is in Danish but there is a detailed summary in English. A description is given of one example of a weak A which *Gammelgaard* calls A_x. The red cells of the A_x donor were said to be agglutinated by anti-A about as weakly as A_5B—which must be virtually negative; furthermore the cells did not absorb anti-A. Yet his saliva contained A substance comparable in amount to that found in A_1 and A_2 people. The parents of the A_x person were dead but five sibs were tested and none of them had the peculiarity.

In 1956 *Wiener* and *Gordon* [9] described a man whose red cells were not agglutinated by anti-A nor anti-B but whose serum contained anti-B only. On testing his saliva A antigen was found. The pheno-

type was called A_m: the letter m stood for monkey. (In 1942 *Wiener*, *Candela* and *Goss* [8] reported that monkeys had B- or A-like antigens in their saliva which were not to be found in their red cells.) *Wiener* and *Gordon* based their opinion that the red cells of the A_m person lack A antigen on agglutination tests with anti-A. If anti-A absorption or elution tests were done they were not reported. No investigation of the genetics of the condition is reported.

The present paper describes the finding of two examples of what is probably the same phenomenon, though the serological results differ slightly from those of *Gammelgaard* and go further than those of *Wiener* and *Gordon*. Fortunately the relatives of both propositi were very willing to help in the investigation.

It is convenient to have a phenotype symbol and we propose to use A_m. *Gammelgaard*'s symbol has priority but will not serve, for *Fischer* and *Hahn* [3] had used A_x in 1935 in their excellent description of the type of blood which has since been variously called A_x, A_4, A_o and A_z. The suffix *m* can more appropriately be taken to stand for modified than for monkey and no offence will be caused to the donors who will certainly want to know what their m means. If further investigation of the A_m of *Wiener* and *Gordon* shows that it is not due to the *yy* genes then the phenotype described in this paper could conveniently be called A_y.

The ABH Antigens of the Two Propositi

The propositus of the first family (Co.) is a blood donor. His red cells appeared by ordinary agglutination tests to be group O but his serum, though it contained anti-B, lacked the expected anti-A. His saliva was then tested and, surprisingly, it contained A substance; it also contained H substance.

The propositus of the second family (Hi.) attended an ante-natal clinic. Her red cells appeared by ordinary agglutination tests to be group B but her serum lacked the expected anti-A. Her saliva was then tested and it too contained A substance; it also contained B and H substance.

Agglutination Tests

Suspensions in saline of the red cells of the two propositi failed to be agglutinated by more than a dozen powerful anti-A sera from group B donors; they failed to be agglutinated by a powerful anti-A

made in a rabbit, and by an extract of the seeds of *Phaseolus lunatus*. Those of Mr. Co. failed to be agglutinated by more than 40 anti-A+B sera from group O donors selected for powerful anti-A (one of these was from a group O recipient accidentally immunized by a transfusion of group A blood).

The agglutination tests were tried at various temperatures and with various dilutions of serum without effect. Suspending the cells in albumin, or treating them with trypsin, papain or ficin failed to make them agglutinable by anti-A. The indirect anti-globulin test, using various dilutions of several anti-globulin sera, was negative. The addition of complement, in the form of fresh AB serum, failed to make the cells agglutinable by anti-A: so did the addition of a small amount of N/10 HCl.

The B antigen of the red cells of Mrs. Hi. is not modified: in agglutination tests against titrations of anti-B the A_mB cells of Mrs. Hi. reacted a little more strongly than did the A_1B cells of her mother or the A_1B cells of her sister. (This difference is seen more strikingly in absorptive power—see below.)

Nor is the H antigen of Mr. Co. or Mrs. Hi. modified: in tests against titrations of human anti-H the A_m cells of Mr. Co. reacted more strongly than A_1 and A_2 controls and nearly as strongly as the O control; against titrations of an extract of the seeds of *Ulex europaeus* the A_mB cells of Mrs. Hi. reacted more strongly than A_1B cells and about the same as the B control. (Here we meet with the only difficult discrepancy between *Gammelgaard*'s results and our own. *Gammelgaard* says his A_x was not agglutinated "by anti-O containing ox serum": we would have expected this serum to give the reactions of anti-H.)

Nothing abnormal was noticed in the agglutination of the cells of the two propositi by antibodies belonging to the other blood group systems: that is to say there was no hint that other antigens were weak; nor did the family groups suggest that the propositi were lacking in antigens for which they might have been expected to have genes.

Absorption and Elution Tests

The red cells of Mr. Co. though not agglutinated by anti-A evidently combine with it for, as shown in table 1, these A_m cells are capable of absorbing anti-A almost as well as A_2 cells and better than A_2B cells.

If the A_mB cells of Mrs. Hi. absorb any anti-A our tests do not show it. Presumably the absorptive power of A_m is greatly reduced by the presence of B—just as that of A_2 is reduced by the presence of B (which is well illustrated in table 1).

TABLE 1

Showing that the A_m Cells of Mr. Co. Absorb Anti-A but that the A_mB Cells of Mrs. Hi. Do Not

Anti-A diluted ¼ absorbed by these red cells	Titration scores against A_1 cells	Titration scores against A_2 cells
A_1	0	0
A_2	11	0
A_m Mr. Co.	15	2
A_2B	31	3
A_mB Mrs. Hi.	43	31
O	45	32
unabsorbed	46	30

Elution tests confirm the combination of A_m with anti-A. The red cells of Mr. Co. after incubation at 4° C. with an excess of anti-A were washed four times in cold saline. A small volume of saline was added to the packed cells and they were heated to between 56° and 60° C. for five minutes. The saline was separated by centrifuging in heated centrifuge cups and it was found to contain quite strong anti-A. As one control the cells were exposed to an artificial mixture of anti-A and anti-B: anti-A but no anti-B was eluted. As another control the cells were exposed to an excess of anti-B: no antibody was eluted. As yet another control O cells were treated with the excess of anti-A: no antibody could be eluted. Anti-A could also be eluted from the A_m cells by the method devised by one of us (*Weiner*[7], 1957).

The A_mB red cells of Mrs. Hi. after exposure to anti-A gave up no antibody on elution, thus confirming the negative absorption results.

No antibody could be eluted from the untreated red cells of Mr. Co. or Mrs. Hi.

Absorption tests with anti-B gave further evidence that the B antigen of Mrs. Hi. was not modified but was, on the contrary, of the

strength expected of a B rather than an A_1B: her A_mB cells were able to absorb more anti-B than were the A_1B cells of her sister.

Other Tests

The red cells of Mr. Co. were not haemolysed by a group O serum capable of lysing A_1, A_2 and B cells.

Two other attempts were made to make the cells agglutinate when combined with anti-A: (1) The cells of Mr. Co. were sensitized with anti-A, washed, and normal A_1 cells were then added to them—but nothing happened. This was done in case the bar to agglutination characteristic of A_m could be overcome if only one of a pair of A cells was subject to it. (2) With the inagglutinable ox cells of *Coombs*, *Gleeson-White* and *Hall*[1] (1951) in mind an attempt was made to bridge the gap between sensitized cells with a chain of anti-A, A substance, anti-A links—but this did not succeed.

One pint of A_m blood from Mr. Co. has been given successfully to an O recipient. There was no adverse reaction nor was there any bio-chemical evidence of intravascular haemolysis (Schumm's test negative, no increase of urobilinogen); furthermore the donor's cells could be seen surviving in the patient's blood one week later and the direct anti-globulin test was negative.

Secretion Tests on Saliva

The saliva of Mr. Co. and of Mrs. Hi. both contain a large quantity of A substance, but it seems that there has been some modification of this too, for in table 2 the amount of A is seen to be less than that found in various control samples. Again the modification is confined to A: the H antigen of Mr. Co. and the B and H of Mrs. Hi. are as strong as those of appropriate controls. (This serves as a useful check on the state of preservation of the samples.) The modification of A was not found in the saliva of the A_m person described by *Wiener* and *Gordon* who say "In fact, the inhibition titre of the patient's saliva was more than four times as high as the inhibition titre of a known secretor of subgroup A_1".

The ABH Antigens of the Two Families

The ABO groups and the secretion results of the families of the two propositi are given in figs. 1 and 2; fortunately they show clearly

TABLE 2

The Amount of A, B and H Substance in the Saliva of the two A_m People Compared with that of Certain Controls

Saliva from	Substance in saliva		
	A	B	H
A_1 secretors (3)	80± 5.3	0	31±11.2
A_1B secretors (4)	73±11.0	55±3.7	6± 6.4
A_2 secretor (1)	60	0	34
A_2B secretor (1)	62	87	29
B secretors (7)	0	64±13.6	16±15.2
O secretors (5)	0	0	56± 7.8
A_m secretor Mr. Co.	24	0	39
A_mB secretor Mrs. Hi.	34	67	17
O non-secretor (1)	0	0	0

The controls include saliva from relatives of the two propositi.

The figures in the columns can be compared with each other, but not those in the rows.

The figures were reached in the following way: titrations of saliva were made in saline, undiluted saliva in the first tube, saliva 1 in 256 in the ninth (and last) tube; an equal volume of anti-A serum (diluted 1 in 2 with saline) was added to each tube and, after mixing, an equal volume of A_2 cell suspension. The agglutination was recorded after one hour, thus:

Saliva	undil.	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
O sec.	+++	+++	+++	+++	+++	+++	+++	+++	+++
A_1B sec.	—	—	—	—	w	(+)	+	+++	+++

Scores were given to the reactions: +++ = 10, ++ = 8, + = 5, (+) = 3, w = 2. The saliva without A scores 90, the A_1B saliva scores 30. The amount of A in the O saliva is taken as 90—90, or 0; the amount of A in the A_1B saliva is taken as 90—30, or 60.

Tests for B substance were done in the same way using an anti-B serum diluted 1 in 2, and B cells- tests for H substance were made with an extract of the seeds of *Ulex europaeus*, and O cells. In our inhibition tests we use anti-A and anti-B sera from donors who have not been stimulated with injections of A or B substance: stimulated antisera are probably less sensitive in detecting antigen by inhibition.

that the genotype of Mr. Co. (II-1, fig. 1) is A_1O and that of Mrs. Hi. (II-6, fig. 2) is A_1B.

Red cells from most of the family members were also tested for the MNSs, P, Rh, Lutheran, Kell, Lewis and Duffy groups. There was nothing remarkable about these groups but they are given in

table 3 in case linkage or some other association between one of their genes and the new modifying gene should later be suspected.

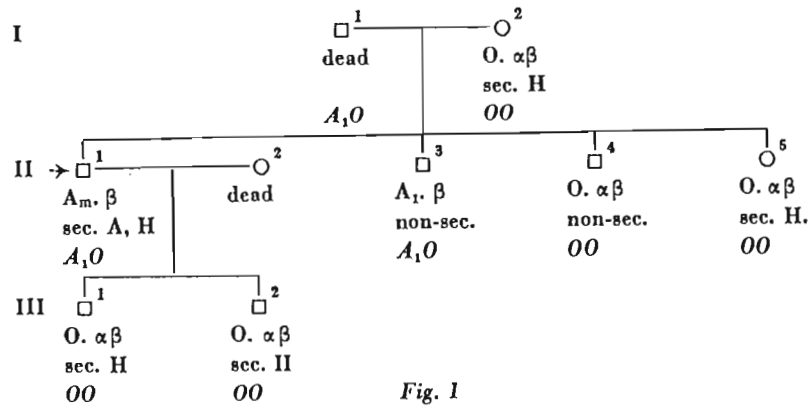

Fig. 1

Fig. 1. The ABO groups of the family Co. showing that the A_m propositus, Mr. Co., II-1, must be of the genotype A_1O. All the cell samples were agglutinated by anti-H and all saliva samples contained Le^a substance save that from III-2.

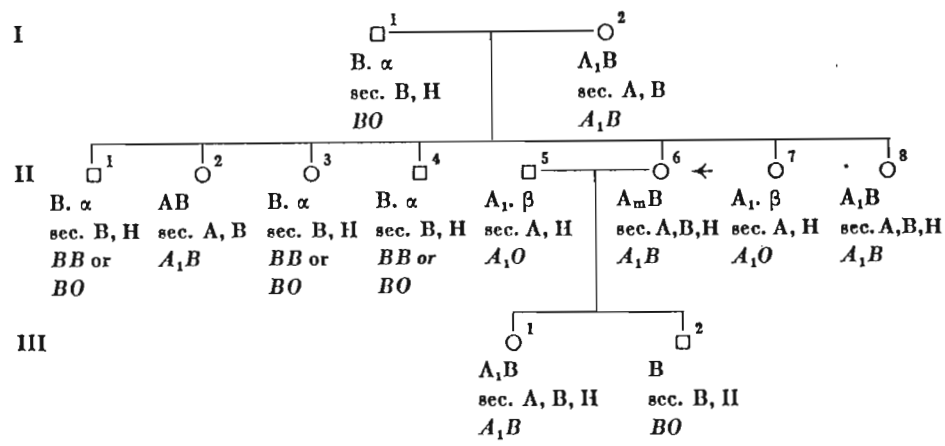

Fig. 2

Fig. 2. The ABO groups of the family Hi. showing that the A_mB propositus, Mrs. Hi., II-6, must be of the genotype A_1B. The saliva of I-2 contained no detectable H substance, that of II-2 was not tested for H or Le^a substance. All other samples contained Le^a substance, save that from II-4. The red cells of II-2 were not tested with a_1 or with anti-H; all other samples were agglutinated by anti-H. No a was detectable in the serum of III-2 but the sample of blood was from the umbilical cord.

TABLE 3

The Blood Groups of the Two Families in Which the Phenotype A_m Occurs

Family Co. (fig. 1)	ABO	MNSs	P	Rh	Lu^a	K	Le^a	Fy^a
I-2	O	*NSNs*	+	*CDe/cDE*	—	—	—	+
II-1	A_m	*NSNs*	+	*CDe/cDE*	—	—	—	+
II-3	A_1	*MN S*	+	*cDE/cde*	—	—	+	+
II-4	O	*NsNs*	+	*CDe/cDE*	—	—	+	+
II-5	O	*NSNs*	+	*CDe/cde*	—	—	—	+
III-1	O	*NN.S*	+	*CDe/cde*	—	—	—	—
III-2	O	*NN.S*	+	*CDe/CDe*	—	—	—	—
Family Hi. (fig. 2)								
I-1	B	*NsNs*	+	*CDe/cde*	—	—	—	+
I-2	A_1B	*MsNs*	+	*CDe/cde*	—	—	—	+
II-1	B	*NsNs*	+	*CDe/cde*	—	—	—	+
II-2	AB	.....	..	.....	..	..	..	..
II-3	B	*NsNs*	+	*CDe/cde*	—	—	—	+
II-4	B	*MsNs*	+	*CDe/cde*	—	—	—	+
II-5	A_1	*MsNs*	+	*CDe/cDE*	—	—	—	—
II-6	A_mB	*MsNs*	+	*CDe/cde*	—	—	—	+
II-7	A_1	*NsNs*	+	*cde/cde*	—	—	—	+
II-8	A_1B	*NsNs*	+	*CDe/cde*	—	—	—	+
III-1	A_1B	*MsNs*	+	*CDe/CDe*	—	..	..	..
III-2	B	.....	..	.....	..	..	..	..

Discussion

The absence of agglutinable A from the red cells of people who have the gene *A* must be an extremely rare event. In routine tests such bloods disclose themselves as examples of "missing" anti-A. The two here reported were the only two found at Birmingham in appropriate tests on between a half and three-quarters of a million samples. (Another cause of missing agglutinins which could easily be distinguished is agammaglobulinaemia: one of our propositi had normal anti-B and both were shown, by paper electrophoresis, to have normal amounts of gamma globulin.)

We assume that the condition is genetically controlled—like everything else we know about blood group antigens: the condition can hardly be due to disease because both propositi are healthy and one of them has normal B and both of them have normal H antigens on their red cells.

Several possible simple genetic backgrounds for A_m suggest themselves and all but one of these are made to seem unlikely by the families Co. and Hi. The most important details of the two families are given in figs. 1 and 2.

1. An Allele of ABO

An allele of *ABO* might have caused A_m: this is excluded because in family Hi. (fig. 2) neither the father nor the mother of the A_mB propositus is A_m. Her mother is a normal A_1B and her father is a normal B: though he is a secretor he does not secrete A as he should have done were he A_mB.

2. A Modifying Gene Dominant in Effect

A_m might be a dominant effect of a modifying gene, say *Y*, acting on a normal *A* gene. Such a gene in family Hi. would have to come from the father, I-1, since the mother is a normal A_1B.

(a) Such a gene could certainly not be closely linked to the *ABO* locus because in family Hi. II-6, the A_mB propositus, must have the same *ABO* genes as her two sibs, II-2 and II-8, who are not A_mB.

(b) An unlinked modifying gene, dominant in its effect, is excluded at the 1 in 32 level of probability: in the two families, and not counting the propositi, there are five people who have A and who would have a 50:50 chance of having also the dominant modifying gene—but none of them were A_m. (The five are: family Co. II-3; family Hi. II-2-7-8 and III-1.)

3. A Modifying Gene Recessive in Its Effect

A_m might be the recessive effect of a modifying gene, say *y*, acting on a normal *A* gene (or, what could scarcely be distinguished, the effect of the absence of a gene *Y* dominant in effect).

(a) Such a gene could not be closely linked to the *ABO* locus for the reasons given in 2 (a).

(b) We think a modifying gene, unlinked to *ABO*, and recessive in its effect is the most likely explanation, and we were disappointed

that in neither family were the parents of the propositi cousins. Slightly in favour of a recessive modification is the precedent of non-secretion and of the modification shown by *Levine* et al. [5] to be responsible for the "Bombay" type of blood.

This is the only simple genetic explanation that fits the two families. If this explanation be true we would expect one quarter of the A sibs of the propositi to be A_m: there are four A sibs and none of them is A_m.

4. Other Simple Possibilities

(a) A_m is not the effect of a peculiar *B* or *O* gene acting on a normal *A* gene: this is excluded because in family Hi. II-6, the A_mB propositus, must have the same *ABO* genes as her two sibs, II-2 and II-8, who wear their A on their red cells in the ordinary way.

(b) Mutation should perhaps be considered as a possible cause of A_m because of the huge numbers out of which the two examples were picked. Since, however, the condition obviously confers no serious disadvantage to survival or fertility inherited examples must greatly outnumber those resulting from new mutations and consequently the chances must be greatly against either of our propositi being mutant. (It is possible that the condition is advantageous: whatever other effects the modifying genes may have the suppression of A_1 on the red cells must protect against ABO haemolytic disease.)

The Effect of the Yy Genes in Different ABO Genotypes

The genotype *yy* affects the genotype A_1O (of Mr. Co.) so that his red cells have modified A which will not agglutinate with anti-A but will nevertheless combine with it.

The genotype *yy* affects the genotype A_1B (of Mrs. Hi.) so that her red cells have no detectable A. This is to be expected because of the known weakness of the antigen A_1 in A_1B cells and A_2 in A_2B cells.

It is reasonable to suppose that the genotype *yy* affects the gene A_2 as well as A_1. If so, A_2 should be at least as severely modified as the A_1 in A_1B people. The negative absorption tests reported by *Gammelgaard* could be explained if his propositus was of the genotype A_2O. This he probably was: though both parents were dead and his genotype could not absolutely be fixed it was very probably A_2O for he had three sibs group O and two group A_2. It is not possible

to guess the *ABO* genotype of *Wiener* and *Gordon*'s example because no absorption test or family investigation was reported.

The two families here reported do not show whether there is any relation between the *Yy* genes and the secretor genes. It is possible, though rather unlikely, that *y* is an allele of *S* and *s*: this would be excluded if an A_m non-secretor were found. To be recognizable such a person would have to be of the genotype A_1O, or A_1A_1, so that the A_m could be detected by absorption and elution tests after the missing anti-A had drawn attention to the blood in the first place. It is rather unlikely that *y* is an allele of *Ss* because the secretor genes have to do with all three antigens, the *Yy* genes with only one.

If the *Yy* and *Ss* genes are independent then a person of the genotype A_2O, *yy*, *ss* would probably be indistinguishable from an O: for, lacking A in red cells and saliva, his serum could reasonably be expected to contain anti-A. (Far fetched as it may seem, this appears to offer the best explanation so far of the famous *Haselhorst* [4] case of a family in which the mother was A_2B, the father O and the child

TABLE 4

Modification of the Expression of the A and B and O Genes as Antigens in Red Cells and in Saliva

Modification		Expression A, B and H antigens	
Phenotype	Genotype	in red cells	in saliva
secretor	*SS* or *Ss*	*expressed*	*expressed*
non-secretor	*ss* (or *S* absent)	*expressed*	*inhibited* A, B and H
"Bombay"	*xx* (or *X* absent)	*inhibited* B and H, no information about A	*inhibited* B and H, no information about A
A_m	*yy* (or *Y* absent)	*inhibited* A only, not B nor H	*expressed*

There is uncertainty about the relationship of the hypothetical gene *O* to the antigen H.

O with anti-A in his serum: the child could have been A_2O, *yy*, *ss*. *Gammelgaard* considered his A_x as a possible explanation of the *Haselhorst* case but decided that the presence of anti-A in the child excluded it.)

The gene *y* in single dose has no apparent effect: the parents of the two propositi who must all be heterozygous *Yy* have normal ABO groups.

The Yy Genes and Other Modifiers of the ABO Genes

The known modifications of the expression of the *ABO* genes as antigens can be summarized in one table (table 4). It is tempting to try to fit the tidy pattern into one genetical system, but one great hindrance to finding a reasonably simple genetic theory to embrace all the known modifications is the fact that A_mB red cells have only their A antigen modified and not their B nor their H, while "Bombay" red cells have both their B and their H modified. It looks as if, so far, we know a part only of a larger pattern of modifications of the ABH antigens.

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Summary

Evidence is given for the existence in man of a locus *Yy*. The very rare genotype *yy* modifies the development of the A antigen in the red cells and, to a very much less extent, in the saliva; the B and H antigens are not affected. The *Yy* locus is not closely linked to the *ABO* locus.

Résumé

Les études sérologiques et génétiques présentées dans ce travail font penser qu'il existe chez l'homme une paire de gènes *Yy*; le rare génotype *yy* influencerait la puissance du facteur A dans les érythrocytes, beaucoup moins dans la salive, tandis que les facteurs B et H

ne sont pas affectés. Il n'y a point de conjugaison étroite entre le locus *Yy* et le locus *ABO*.

Zusammenfassung

Auf Grund der vorliegenden genetischen und serologischen Untersuchungen ist anzunehmen, daß beim Menschen ein Genpaar *Yy* vorkommt, wobei der sehr seltene Genotypus *yy* die phänotypische Ausprägung des Blutgruppenantigens A in den Erythrozyten beeinflußt. Die Ausscheidung von Blutgruppensubstanz A im Speichel wird kaum beeinflußt. Der Genotypus *yy* hat keinen Einfluß auf die phänotypische Ausprägung der Antigene B und H. Der Genlokus *Yy* zeigt keine enge Koppelung mit dem Genlokus *ABO*.

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Authors' addresses:
Dr. *W. Weiner*, Blood Transfusion Service, 15 Ampton Road, Edgbaston, *Birmingham 15;*
Dr. *R. R. Race*, Medical Research Council, Blood Group Research Unit, The Lister Institute, Chelsea Bridge Road, *London, S. W.* 1 (England).

Paper 11

THE A SUBGROUPS OF THE ABO BLOOD GROUP SYSTEM

PHYLLIS P. MOORES, B.Sc.
Natal Blood Transfusion Service, Durban

The ABO blood groups were discovered by Dr. Karl Landsteiner in 1900. While mixing the cells of some of his co-workers with the serum of others, he noticed that they could be divided into three distinct categories or groups to which he gave the names A, B and O. Shortly afterwards a fourth group — AB was added.

In 1911 von Dungern and Hirszfeld[4] recognised that there were certain differences in the A group which led Landsteiner and Levine[21] subsequently to the identification of two subgroups A_1 and A_2.

In the years that followed many further subgroups of A were identified. Unfortunately however, owing to the simultaneous description of similar subgroup examples under different descriptive names, it was not long before there was a good deal of confusion. As news of the confusion spread some of the original workers changed the names of their subgroups, only to find in due course that the new one they had chosen was already in use elsewhere! Recognising the rapidly deterioriating state of affairs Race[25] made a firm attempt to generalise the wealth of detail into two reasonably crisply defined groups under the heading 'Weak A' which he called A_x and A_m. This theory enjoyed a period of strong support until fairly recently, but investigators are now beginning to look beyond the confines of Caucasians. Some new subgroups which seem to be special to certain Bantu and Negroid populations have been reported and do not fit into the scheme.

The purpose of this paper is to present the principal characteristics of as many of the subgroups of A as possible as they are known at present, so that the information can be studied on a comparative basis from one article. It is hoped that this will be of some benefit to the modern blood group serologist who would rather like to know the specific identity of the examples which come his way during the course of his work, but who is overwhelmed by the wealth of information contained in numerous articles from many different journals.

No challenge is intended against the very worthy attempts made to bring about simplification; rather the points which support this will be emphasised.

The following subgroups of A will be considered in the text in parallel as far as possible:

A_1, A_2, A_3, A_4, A_5, A_6, A_z, A_x, A_o, C, A_m, A_g, A_{end}, A_{el}, A_1^w, A_3^w, A_{int}, A_{bantu}, and the modifications which have been brought about by disease

chimerism; inhibitor, suppressor and expressor genes, and the suppression effects of the blood group B. A composite table will be drawn finally for easy reference.

Brief Historical Background

The origin of subgroups A_1 and A_2 may be read again from the introduction.

The subgroup A_3 is considered by most authorities to be a separate distinct unity which would probably be grouped similarly by most workers no matter how their techniques might vary. It was first recognised by Friedenreich in 1936.[9] Examples identified independently and reported by Fischer and Hahn,[8] were later shown not be be A_3, but A_4 by Gammelgaard in 1942,[11] who wrote a thesis in Danish, (fortunately an English translation has recently been published) in which he studied the A_3 subgroup in 203 members of 26 families, adding much important detail.

Gammelgaard also examined 10 examples of A_4, and 27 A_5's in his thesis and in doing so realigned O. Hartman's A_3[17] as an A_5.

Workers have noted that certain subsequently discovered A_4's differ slightly from Gammelgaard's description.

A_5, Jonsson's A_6[19] and Estola and Elo's A_z[7] have been reported by Glover and Walford[12] to be very much the same, but, whereas Race and Sanger[25] place A_5 and A_z in the same category as A_4, A_o and A_x, under the general heading A_x, Glover and Walford consider that only A_4, A_o and A_x qualify for this simplification. Fischer and Hahn's A_x[8] is quite clear and recognisable as an A, but certain serum variations were found by Jakobowicz,[18] and Dunsford.[5] Grove-Rasmussen's A_o[15] however, found in 1952, looked very much more like an 0. Wiener's group C,[34] published in 1953 was remarkable not so much for the character of its cells which can be placed under Race and Sanger's general heading A_x, as for the postulation that most group O sera contain not only anti-A and anti-B, but an anti-C which is the agent responsible for the specific agglutination of these cells. Other workers believe a cross-reacting anti-A + B to be the cause.

The next two subgroups A_m and A_g were found both in persons suffering from chronic myeloid leukaemia and myeloblastic leukaemia respectively, and in perfectly normal people. Weiner and Gordon's A_m[35] at first seemed similar to an O, but Van Loghem and Van der Hart's A_g[23] was more A-like. A_m has also been shown to be the result of the action of a suppressor gene y.[32]

By this time everyone was confused and in reporting a new variety of subgroup Weiner, Sanger and Race[33] refused to name it at all. However, more examples of it were soon found and Sturgeon, Moore and Weiner[28] finally gave it the name A_{end} using some of the letters of the name of the first person in whom it was discovered. This was followed by Sturgeon's

A_{el}[28, 27] in which the subscript denotes that the only A-like property of the cells is discernable in eluates.

A_1^w of Gold and Dunsford[14] echoed A_3 in some of its characteristics, but was thought to be the effect of suppressor and expressor genes with incomplete penetrance of the expressor or dominance of the suppressor so that only a very small quantity of A_1 was present in the cells. There was no history of chronic disease.

Unfortunately the details of A_3^{w24} were not available to the author at the time of this publication.

A subgroup that had been recognised but not understood in Caucasians and Asiatics has recently been brought to the forefront through the work of Brain on South African Bantu[3]. This is A_{int} which is intermediate in A antigen strength between A_1 and A_2; the red cells, however, have almost as much H substance as O cells. Brain's A_{bantu} however, seems so far to be a characteristic of the Bantu alone, and has an A_3-like appearance.

When the presence of a subgroup of A is suspected, modifications that may have been brought about by outside causes must always be taken into consideration. It is very easy to be misled into interpreting a result as weak A in instances where an A person has received a transfusion of O blood, the foetus has bled back into the mother in utero, or where it can be shown that the subject is an A/O blood group chimera,[2] and these factors should first be excluded. Secondly, cases of chronic leukaemia have been associated with changes in the A group which seems to have been altered in stages to an O-like appearance.[13] 'Bombay' x genes[1, 26] are capable of suppressing the expression of the blood group entirely, and y genes can effectively modify an A_1 group to A_m[32]. In these instances it is usual for the next generation to inherit a normal group. Finally, the depressing effects of an associated group B have been well verified in the literature.[25, 29] Most A's in the combination AB look like weaker forms of A and may mislead the investigator into believing that they are actual examples of an A subgroup.

Agglutination with the Anti-A of Group B Serum

Characteristically the strength of the agglutination of a weak A with anti-A of group B serum is not associated with the titre of that serum. A_1, A_2 and A_{int} are almost always strongly agglutinated, A_1 usually more so than A_2, and A_{int} in between; though the difference between A_1 and A_2 is thought to be only qualitative. When the agglutinates are examined under the microscope the stronger the A the fewer the free or unagglutinated cells that are visible, but this never reaches the extent to which it does for A_3. The agglutination of A_3's is very characteristic. A few not very small but fairly fragile agglutinates are visible in a veritable sea of unagglutinated cells. Some other subgroups present the same type of mixed field appearance. These are A_g, which may also be negative, A_{end}

which only produces the effect by the more sensitive petri-dish method of Race and Sanger[2], A_1^w in which the agglutination is very feeble, A_3^w — presumably since this is a characteristic for A_3, A_{bantu} and A/O blood group chimera's. In Gammelgaard's A_4 and A_5, and in A_6 the agglutination, though weak, is general, his A_4 being slightly stronger than A_5 and A_6. A_5 cells, however, also exhibit an unusual phenomenom in that examples are usually agglutinated less strongly by the more powerful, or higher titred anti-A's than by the standard type. A_6, A_x Group C[31] and changes due to leukaemia, if they are agglutinated at all, will only be very weakly so by a very few standard anti-A (Group B) sera much to the same degree that the cells of an A_5B would be. Jakobowicz's A_x, however, was agglutinated by 8 out of 17 samples.

Other A_4, A_z, A_o, A_m, some A_g's, A_{end} by tube technique and A_{el}, are all negative with anti-A. Persons under the effect of Bombay x, or y genes also give negative results with anti-A, while those with a B group acting as a depressor have reduced agglutination. The weaker the basic A type, the weaker the effect will be when it is linked with group B. An unusual person has been reported by Furuhata, Kithama and Nozawa[10] who seemed to have cells some of which were A_1 some A_2 and some weak A but the serological details are not known.

Agglutination with the Anti-A of Group O Serum

Although the weak A's are often characterised by the way in which they react with the anti-A of group B serum it is the anti-A of group O serum which distinguishes most of them as belonging in group A, and the type of agglutination is usually stronger. A_1, A_2, A_{int}, Gammelgaard's A_4, other A_4, A_5, A_6, A_x[30], A_o and Group C are all agglutinated, some more powerfully than others, with few or more free cells, but all with an overall general pattern of agglutination. A_3 gives the same characteristic mixed field appearance as it did with anti-A (Group B) sera, but there are some anti-A (Group O) samples in existence which will agglutinate all the cells, proving conclusively that this type of blood is not just a simple mixture or mosaic of A_2 and O cells. Another unusual feature is that A_3 cells will agglutinate in higher dilutions of anti-A (Group O) sera than the cells of an A_2B, the degree of agglutination only decreasing very slightly as the test serum dilution increases.

The A_3 mixed field type of agglutination can be seen with A_{end} cells but only when the sensitive petri-dish method is used; with A_1^w cells but for only 50% of the sera used and with results no stronger than those for anti-A (Group B) sera; with A_3^w cells presumably, with A_{bantu} definitely and for A/O blood group chimera's always. Anti-A (Group O) serum will not agglutinate A_m, A_{el} or cells under the effects of Bombay suppressor x or modifier y genes, and is negative or only very weakly positive with A_g and A's modified by leukaemic changes. There are no results available for A_z. Unfortunately the presence of the B gene made observation of its

depressive effect on an A impossible in this instance, since Group O serum also agglutinates Group B cells.

Agglutination with Immune Anti-A Serum

This type of anti-A is considered to have special properties of its own, and may be derived from the inoculation of rabbits with group A cells, from the serum of a patient who has had a transfusion reaction, or from mothers who have given birth to an infant suffering from haemolytic disease of the newborn due to anti-A. It is used as a descriptive tool in the study of the weaker forms of group A. A_1, A_2 and A_{int} are agglutinated strongly, but the A_2 agglutinates will show more than just a few unagglutinated cells. A_{bantu} appears positive to the naked eye but presents a mixed field appearance microscopically, while A_3 cells are only weakly agglutinated, their antigenic strength actually being less than that for an A_2B. A_4, A_5, A_x Group C and some cells with leukaemic changes, are all weakly agglutinated. A_o, A_m, A_{el} cells under the effects of x and y genes, and others with leukaemic changes, are not agglutinated, while A_g, A_{end} in petri-dishes, A_1^w and A/O chimeras are all agglutinated with the typical mixed field appearance. One special immune anti-A has been described which agglutinated all the cells of an A_{end} when the indirect antiglobulin technique was used. There was unfortunately no record of the effect on A_6, A_z or A_3^w cells available to the author. The agglutination of group B-modified A's is usually stronger than with the standard anti-A of Group B sera.

Agglutination with Anti-A_1 Sera

Anti-A_1 may be of human or plant seed origin, seed extracts such as those from Dolichos biflorus providing most of the stronger samples. The seed extracts have been much used in separating or attempting to separate A cells from suspected mixtures of A and O; the advantage lying in the comparative ease with which the seed extract agglutination can be reversed by the addition of blood group specific substances.[2]

A_1 exhibits strong agglutination. A_2, A_3, A_4, A_5, A_z, A_x, A_o Group C A_m, A_g, A_{end}, A_{el}, A_{bantu}, leukaemic changes of A and cells under the effects of genes x and y are all negative. A_1^w gives feeble agglutination, with a mixed field picture, and A/O chimeras will do the same providing their A is A_1 and not one of the subgroups above. A_{int} and A_1B's will agglutinate with anti-A_1 but more weakly than A_1 cells, and there is no record for A_6 or A_3^w.

Agglutination with Anti-H

Although examples of human anti-H are known the kind most usualle used for the investigation of weak A's is obtained in extracts from thy seeds of the plant Ulex europeus.

In Caucasians it is an acknowledged fact that the weaker the A the stronger its reactions with anti-H. The strongest results are in fact obtained

TABLE OF THE GENERAL CHARACTERISTICS OF THE SUBGROUPS OF A

					Race's A_x								Race's A_m												
	A_1	A_{int}	A_2	A_3	Gammel-Gards A_4	A_4	A_5	A_6	A_z	A_x	A_o	C	A_m	A_g	A_{end}	A_{el}	$A_1{}^w$	$A_3{}^w$	A_{bantu}	Leukemic Changes	A/O Chimerism	Bombay x	Modifying y	Depressive B
Anti-A (Group B)	÷	+	÷	MF	(÷)	—	(÷)	(—) —	—	(+) —	—	(+) —	—	MF —	MF* —	—	MF	occ MF	MF	(+) —	MF	—	—	REDUCED
Anti-A (Group O)	÷	+	÷	MF	+	—	÷	÷		+	÷	+	—	(+) —	MF*	—	occ MF		MF	(+) —	MF	—	—	+
Immune Anti-A	÷	+	÷	(÷)	(÷)	(—)	(÷)			(÷)	—	(+)	—	MF	MF*	—	MF		MF	(+) —	MF	—	—	+
Anti-A_1	÷	÷	—	—	—	—	—		—	—	—	—	—	—	—	—	(MF)		—	—	MF if A_1/O	—	—	(÷) if A_1B
Anti-H Caucasian	(—) —	++	÷	÷	÷	—	÷		÷	÷	÷	+	+	÷	+	÷	÷			÷	÷	—	+	(÷)
Bantu	÷ —	++	÷																++					
Absorbtion of Anti-A	÷	+	÷	+	+	÷	(÷)		÷	+	+	+	+	+	+	+	÷		+	+	+	—	+	REDUCED
Elution of Anti-A	(÷)	(÷)	÷	+	+	—	÷		÷	÷	+	+	+	+	(+)	+	(÷)		+	+	Poss (+)	—	+	REDUCED
Saliva Substances A	+	+	÷	(÷)	(+)	(—)	(÷)		(—)	(÷) ++	(÷)	(+)	+	+	(+)	(+)	non-sec.		(+)	+	+	usually all non-sec.	÷	+
H	÷	+	+	+	÷	÷	÷		÷	÷	+	÷	(+)		+	+			+	+	+		(+)	(+)
Serum Anti-A_1	—	—	some	some	some	some	÷		÷	÷	÷	÷	—	—	—	+	+		+	some	—	+	—	+
Anti-A	—	—	—	—	some	some	some		+	÷	—	—	—	—	—	—	+		—	some	—	÷	—	—

(+) = Weak Positive Occ = Occasional MF = Mixed field of agglutinated and unagglutinated cells * = in Petri dishes non-sec. = non-secretor

for group O, but Brain has found that the rule does not hold good for the Bantu of South Africa. Though Caucasian A_1's always give negative or very weak results with anti-H Bantu A_1's may give quite strong reactions. Caucasian A_2's are intermediate between A_1 and O for H but again Bantu A_2's often have high H scores. Known Caucasian,[22] Asiatic[36] and Bantu A_{ints} however, are more uniform and all have a great deal of H on their cells irrespective of racial origin. Brain's results have also shown that the mean H level of the A_{bantu} subgroup is quite remarkably high, being possibly even greater than that for group O, and certainly higher than Caucasian bloods of all ABO groups.

A_3, A_4, A_5, A_z, A_x, A_o Group C A_m, A_g, A_{el} and y gene modifications are all agglutinated by anti-H. A_{end} and A_1^w results are in strength approximately midway between those for Caucasian A_2 and O, though A_1^w cells with human anti-H react like O cells. A's modified by leukaemic changes give strong results almost to the level of an O, and A/O blood group chimera results are also good. Only Bombay x suppressed cells give completely negative results with anti-H and this is one of their particular characteristics. Group B modified A's only react weakly, and there is no record available for A_6 or A_3^w.

The Absorption and Elution of Anti-A

Group A's to be worthy of the label 'A' should at least be capable of either absorbing anti-A or allowing it to be eluted from their cells, or both. In the literature it is not always clear however, whether the anti-A from Group B, or from Group O sera, has been used. It seems to be a general observation that the weaker the A the weaker its power to absorb anti-A, until the limit is reached in group O which does not absorb any A at all. By complete contrast, when elutions are made from these same cells, the weaker the A the easier it is to obtain the anti-A again in the eluate.

A_1 and A_{int} absorb anti-A with relative ease but only give it up again with difficulty. A/O chimeras and A_1^w provide almost the same results, and eluates from A_1^w cells are not as strong as those from A_2 cells. A_2, A_3, A_4, A_z, A_x, A_o, Group C A_m, A_{end}, A_{el}, A_{bantu}, leukaemic disease-altered A's and those modified by y genes will all absorb anti-A and give it up on elution, but there are the following differences: A_3 cells absorb more anti-A than A_2B cells because the A antigen in them is thought to be in varying degrees of development. Thus some of them may actually have more A antigen than A_2 cells. A_4 cells absorb less than A_2B, and A_{el} will not absorb any anti-A_1. A_o gives up large amounts of anti-A in eluates from Group O sera but only a little from Group B sera, and A_m cells in the combination A_mB will not give anything in the eluate although A_m by itself will. A_g cells will give up more than A_4 cells, but A_{end} less than A_1, A_2, A_2B, A_3 or A_m. A_5 absorbs hardly any anti-A yet apparently gives it up satisfactorily on elution, and B modified A's show reduced absorption and elution. The author has no records of the reactions of A_6 and A_3^w.

Substances Detectable in the Saliva of Secretors

Of all the tests for weak A, those made on the saliva of secretors have probably proved to be the most valuable when the patient's cell antigen strength approximated that of Group O. To give two instances, the original examples of subgroup group A_m and some of the A's altered by leukaemic changes drew attention to the fact that they were A's through the demonstration of A substances in their saliva. Where the secretion of H is concerned, on the average the more 'O'-like the A, the more H substance will be found, according to the 'pleiades' theory of Hirszfeld[16]. A_1 subjects usually secrete a great deal of A but little or no H, though this can only be said to hold true for Caucasians. A_2's secrete less A and more H, and A_3 less still and more still respectively. A_4 secretes no A or only barest traces, but lots of H, and so do A_5, A_z some A_x's, A_o Group C A_{end}, A_{el} and A_{bantu}. Other A_x's are strong secretors of A, almost as good as A_1 and A_2 and this has led to the belief that A_x is possibly a defective group in which the alcohol-soluble antigen producing system may be being suppressed by a genetically determined modifying gene, while the water-soluble system is normal.[11] On the other hand it may be normal for two varieties of A_x to exist, one with A in the saliva and one without.

A_m, A_g, A_{int} A/O blood group chimeras, B group modified A's and leukaemic modified A's all secrete A in good quantities. A_m and A_{int} secrete some H, but the results for A_g have not been given. No record of secretion for A_6 or A_3^w is known by the author, and the lone example of A_1^w unhappily proved to be a non-secretor.

Presence of Anti-A_1 and Anti-A in the Serum

Variations within the subgroups are common when this factor is under consideration, and little cognisence has been taken of results found. However, for the benefit of completeness they will be included here.

A_1 has not been found with anti-A_1 or anti-A present in the serum. 1-2% of A's and 26% of A_2B's have anti-A_1 but not anti-A. A_{int} does not have anti-A and there is no record of whether or not it can have anti-A_1. A_3 does not usually have anti-A_1, but Dunsford found examples which did[6]. No anti-A is present. A_4, A_5 and Group C are often found with anti-A_1, and A_5 occasionally has anti-A. There is no record for A_6 or A_3^w but A_z and A_x can have both anti-A_1 and anti-A and so does A_1^w though only active at cold temperatures. A_o, A_{el}, A_{bantu} and B modified A's may have anti-A_1 but sometimes this may only be active at cold temperatures. They have no anti-A. A_m, A_g, A_{end}, A/O chimeras and y gene modified people do not have either anti-A_1 or anti-A. Persons having leukaemic modified A groups however, seem generally to be found with both anti-A_1 and anti-A in their serum.

Conclusions

Examination of the details will soon show the reader the value of the

simplifications proposed by Race and Sanger in classifying the subgroups of A. Under the general heading A_x are placed (*a*) all those negative or weak with anti-A (Group B) but giving good reactions with anti-A (Group O); (*b*) having in their serum no anti-A but usually anti-A_1 and (*c*) having saliva which contains H but no A. Under a second general heading A_m are included (*a*) those which are negative or weak with both anti-A (Group B) and anti-A (Group O), (*b*) having no anti-A or anti-A_1 in the serum, and (*c*) having A and H in the saliva. These latter examples usually present as group O with missing anti-A, yet are capable of absorbing anti-A.

Most of the subgroups should be capable of identification if due care is taken.[11] A_3 presents no difficulty as the agglutination is characteristic, but it might be possible to mistake an A_4 for A_2 if only strong and immune anti-A sera are used. A_4 and A_o might also look similar to an O if the grouping sera are too weak. They can have anti-A_1 in the serum which, if A_1 cells have been used in the serum grouping, will make them appear to give the same agglutination reactions as group O. A_5 will never be mistaken for A_2 but could be confused with O for the same reason as A_4. A_x might be mistaken for either A_5 or O but will be distinguished from A_5 by the amount of A antigen in the saliva and the absence of anti-A_1 in the serum. It will not however, be easily distinguished from group O.

As a last resort A_1, A_2, A_3, A_4, A_5 and A_o are all known to have stimulated the production of anti-A when inoculated into rabbits and the serologist may therefore conclude that **antigenicity** may also be used as an additional tool in the determination of the character of a weak A.

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Study 1

Subgroups of A and AB in Natal Negro blood donors

(Pages 43-55 and 205 in M.Sc. Thesis by P.P. Moores. University of Natal, 1976).

Introduction

It was known before Brain's work of 1966 that a wide range of A blood group variability existed in Negroes (Shapiro, 1951a, 1951c) but they were being classified with anti-A_1 reagents only into A_1-positive and A_1-negative (or A_2). Shapiro suggested (1951c) that the frequencies of group AB persons reported by Elsdon-Dew (1936b) in many African tribes were too low, due to the use of low-titred, non-immune anti-A reagents; and the frequent occurrence of anti-A_1 in the serum of A_2 individuals led many workers to use Caucasian A_2 instead of A_1 cells in ABO isoagglutinin tests to avoid inadvertently grouping them as O. Brain (1966) showed that there were four clearly defined subgroups of A in the Natal Negroes: A_1, A_{int}, A_2 and a weak form called A_{bantu}; and that the amount of H antigen on Negro cells could vary from individual to individual even within the same A sub-type.

Negro group A cells were shown by Grace in 1969 in quantitative tests with anti-A extracted from snails (Helix aspersa) to have more A antigen than Caucasoid group A cells, but the snail extract was not able to distinguisn between the various A subtypes. Negro group B cells, studied quantitatively with human anti-B serum by Formaggio in 1953 and by Gibbs, Akeroyd and Zapf in 1961, were shown to have more B antigen than Caucasoid group B cells also, and this was confirmed in the Natal Negroes by Brain and by Milner and Calitz in 1968.

Investigations have shown that a number of group A and AB phenotypes

with variable expression of both A and H antigen exist in Negroes, but no studies of their inheritance have been reported yet (Voak and Lodge, 1970; Ssebabi, 1973). The A_{bantu} phenotype has been found to occur more frequently in the Khoikhoi, and the families examined indicate that it is inherited in them as a normal Mendelian character, recessive to A_1 and A_2 (Jenkins, 1972, 1974). The A_{int} phenotype has unfortunately not been identified in the same way by all investigators (Sathe and Bhatia, 1974).

Materials and Methods

Subgroups of A

The frequencies of the A_1, A_2 and A_{bantu} phenotypes in the Negro and White donors (no attempt was made to determine the A_{int} frequency) were determined in a separate study in which red cells from all the blood samples grouped as A each day for two months by the Services's laboratory staff (from 40 to 80 per day) were first regrouped with anti-A, anti-B and anti-A,B by technique 1 (saline tube test), the tests being centrifuged before they were read to confirm this, and were then tested with a selected potent anti-A_1 reagent which worked very well by technique 2 (bromelin tube test). Those that were agglutinated strongly by the anti-A_1 reagent (4 on the scale of red cell agglutination values given in Chapter II) were considered to be A_1 while those that were weakly agglutinated (possible examples of A_{int}) or negative were retested with anti-A and anti-A,B by technique 1, the results being read this time without prior centrifuging as it was important that they should be examined in this way for the presence of mixed fields of agglutinated and unagglutinated cells

characteristic of A_{bantu} (Brain, 1966). If mixed fields were not detected, i.e. all the cells were agglutinated, they were called "A_2" even though it was realised that this group also contained all the A_{int} samples. The "A_2" and A_{bantu} samples were also tested in titrations (technique 8) with serial doubling dilutions of anti-H reagent (Ulex europaeus) followed by technique 1 (saline tube test) with prior centrifugation to determine their H antigen content in relation to suitable Caucasoid control cells, and their serum was tested against A_1, A_2 and O cells by technique 1 (with centrifugation) for the presence of anti-A_1 isoagglutinins. The anti-H titrations were done to confirm that the samples had more H antigen than Caucasoid group A cells and that the amount of H antigen also varied between individuals of the same A subtype as described by Brain (1966, 1968), but they are not described in detail in the Results section of this chapter. Similarly, the results of the isoagglutinin tests, which were made to assist in determining the phenotypes and to confirm that the majority of the A_{bantu} had anti-A_1 in their plasma (Brain, 1968), have been omitted. Standardised A_1B serum was used as a neutral control reagent with all the tests to avoid the inclusion of false positive results due to auto-agglutination or chance contamination.

Subgroups of AB

As the frequency of "unspecified weak AB" samples among the group AB donors was expected to be higher than the frequency of "unspecified weak A" samples among the group A donors (Voak and Lodge, 1970; Ssebabi, 1973), and no previous results were available for Natal, a

separate study was made of A and H antigen variation in the group AB donors in detail. Blood samples from 3 to 15 group AB donors daily were examined by the same tests used to classify the A subgroup donors, but no attempt was made to place them in categories similar to those of the A subgroup donors as the B gene present was likely to have had a weakening effect on the expression of the A antigen (Young and Witebsky, 1945). Instead they were divided arbitrarily into six subtypes as follows:

(a) strongly agglutinated by anti-A_1 with no evidence of H antigen;

(b) strongly agglutinated by anti-A_1 with H antigen detected in titrations with Ulex anti-H;

(c) weakly agglutinated by anti-A_1 with no evidence of H antigen;

(d) weakly agglutinated by anti-A_1 with H antigen detected in titrations with Ulex anti-H;

(e) no agglutination by anti-A_1 with no evidence of H antigen;

(f) no agglutination by anti-A_1 with H antigen detected in titrations with Ulex anti-H.

Only the presence or absence of H antigen, and not the amount of H antigen detected, was recorded in this investigation in order to avoid further complication; but the frequency with which anti-A_1 isoagglutinins occurred in the serum of the non-A_1B samples was carefully noted.

Results

Subgroups of A

The frequency of "unspecified weak A" samples among the group A Negro

donors in the present study was higher than the frequency reported by the Natal Blood Transfusion Service in 1960 (see Table III.3). This was attributed to more confident recognition in the present study of the A_{bantu} phenotype as a result of the criteria established by Brain in 1966 and to the use of high titre immune grouping sera. The deficiency of AB subjects observed in the N.B.T.S. 1960 series which approaches the conventional level of significance ($p = 0,2$, see Table III.1) suggested that some of the weaker A's, perhaps when combined with B, were missed on this occasion. In support of this view, the "unspecified weak A" group frequency in the present study (Table III.3) was seen to be almost identical with the A_{bantu} frequency in Negroes reported by Brain in 1966 (Table III.4) and with the specific A_{bantu} frequency obtained in a special study of the A subgroups in the Negro group A donors (see Table III.4). The frequency of similar "unspecified weak A" samples found in the White group A donors was very low (see Table III.3), but was in keeping with the virtual absence of A_{bantu} in this race (Race and Sanger, 1968).

The frequencies of the specific A subtypes which were identified in the large sample of group A Negro donors in Durban is shown in Table III.4 for conparison with those found by Brain in 1966. The A_{bantu} frequencies were almost identical, as already mentioned, but the frequency of the "A_2" samples was slightly higher than the frequency of A_2 and A_{int} found by Brain (1966) when these were added together. The frequencies of the specific A subtype samples in the group A White donors was in keeping with those for Whites in Britain (Race

TABLE III.1

ABO Phenotype and Allele Frequency in Natal Negroes Recorded by Other Workers

Tribe or people tested	Area of habitation	Number tested	% Phenotype Frequency				Allele Frequency			AB subjects (calculated to nearest whole No.		X^2	p	Reference
			A	B	O	AB	p	q	r	Observed	Expected			
Negroes (Zulus)	Northern Zululand	500	24,6	21,6	51,8	2,0	0,1441	0,1266	0,7293	10	21	4,3	<0,05	Elsdon-Dew, 1936b
Negroes (Zulus)	Northern Natal	322	33,54	23,29	37,58	5,59	0,2209	0,1575	0,6216	18	25	1,2	<0,3	Zoutendyk, 1955a
Negroes (African women)	Natal	2 526	28,31	20,79	46,33	4,59	0,1811	0,1363	0,6826	116	128	0,84	<0,5	Hirsch, 1958
Negro donors	Natal	3 580	31,64	19,22	44,34	4,80	0,2028	0,1283	0,6659	172	193	1,68	<0,2	Natal Blood Transfusion Service, 1960
Negroes ('Bantu' women)	Natal	7 105	28,45	21,49	45,01	5,05	0,1847	0,1430	0,6723	359	382	1,03	<0,5	Buckwalter et al., 1961
Negroes ('Bantu')	Durban	867	30,7	19,4	43,8	6,1	0,2050	0,1369	0,6618	53	46	0,61	<0,5	Brain, 1966
Negroes (Zulus)	Johannesburg	56	23,2	14,3	53,6	8,9								Elsdon-Dew, 1934a
Pooled Nguni Negroes (Xhosa, Mpondo, Baca, Zulu, Swazi)	Johannesburg	500	29,6	21,8	44,4	4,6	0,1864	0,1398	0,6663	23	29	0,75	<0,5	Elsdon-Dew, 1934a
Negroes (Zulus)	Johannesburg	403	27,0	17,6	50,4	4,9	0,1754	0,1202	0,7099	20	16	0,78	<0,5	Shapiro, 1951c

TABLE III.3

Percentage Frequencies of Unspecified Weak A's and AB's in Group A and Group AB Negro and White Donors in Natal

Tribe or people tested	Area of habitation	Phenotype Frequencies						Reference
		Number tested	A	Weak A	Number tested	AB	Weak AB	
Negro donors ('Bantu')	Natal	1 133	97,09	2,91	172	90,13	9,87	Natal Blood Transfusion Service, 1960
Negro donors	Durban	2 499	95,6	4,40	409	84,84	15,16	Present study
White donors	Durban	1 532	99,94	0,06	147	98,64	1,36	Present study

TABLE III.4

Percentage Frequencies of the Subgroups of A in Negro and White Donors of Group A only in Durban

Tribe or people tested	Area of habitation	Number tested	Phenotype Frequency				Reference
			A_1	A_{int}	A_2	A_{bantu}	
Negro donors ('Bantu')	Durban	219	60,27	13,70	21,92	4,11	Brain, 1966
				Combined Frequency 35,62			
Negro donors	Durban	472	58,48	37,28		4,24	Present study
White donors	Durban	557	75,39	24,60		0,0	Present study

and Sanger, 1968) where the A_{int} frequency is known to be less than 1%.

Subgroups of AB

The frequency of "unspecified weak AB" samples among the group AB Negro donors in the present study was seen to be higher than the frequency reported by the Natal Blood Transfusion Service in 1960 (see Table III.3), possibly for the same reasons as those given for the increase observed in the "unspecified weak A" samples in the group A Negro donors, but the amount of A antigen is also known to vary in group AB's due to the weakening effect caused by the presence of a B gene (Voak and Lodge, 1970; Ssebabi, 1973). This, therefore, may have also been due to chance. The frequency of "unspecified weak AB" samples among the group AB White donors was low and in keeping with the low frequency for Whites in Britain (Race and Sanger, 1968, p 17).

The results of the study which was made of A and H antigen variation in the Negro and White group AB donors are given in Table III.5 where it will be seen that there was a much lower frequency of group A_1B Negroes with no detectable H antigen, designated category a, than in the White donors; while in categories b to f, in which there was either an increased amount of H antigen or a decreased amount or an alternative form of the A antigen shown by a weak or negative result with the anti-A_1 reagent, there was a higher frequency in the Negro than in the White donors. Even when the frequencies in category a and b, and those in c and d, and in e and f, were added together in each race to eliminate the subdivisions introduced by the presence or

TABLE III.5

Variability of A and H Antigen Expression in Group AB Negro and White Donors of Durban

Tribe of people tested	Area of habitation	Number tested	Red cell antigens A_1 : H :	Categories of Group AB Observed					
				a	b	c	d	e	f
				Present [4] Absent	Present [4] Present	Present [1] Absent	Present [1] Present	Absent Absent	Absent Present
Negro donors	Durban	102		10,78	7,84	21,57	10,78	20,59	28,43
White donors	Durban	114		51,76	4,38	14,91	0,87	7,89	20,18

absence of H antigen which Brain had shown in 1966 in any case varied in Negro group A individuals of the same A subtype, this trend was apparent. The results indicated, as expected, that the frequency of Negro group AB's which were agglutinated less than 4 by anti-A_1 or had some H antigen (categories b to f) was higher than in the Whites, and that H antigen could be present or absent on Negro or White group AB cells irrespective of the A subtype, or alternatively of the amount of A antigen present.

Anti-A_1 isoagglutinins were detected in the serum of only 29% of the Negro non-A_1B blood samples.

Discussion

The subgroup A_1 phenotype frequency in the Natal Negroes, in common with the high p allele frequency, was one of the highest recorded in the Negroes, who were all shown in Appendix 2 to have a lower average A_1 frequency than most other peoples in Africa. Their A_2 and A_{bantu} frequencies however, were well within the known high Negro range for these phenotypes. The A_{bantu} phenotype is almost certainly a Negro character, but its much higher frequency in the San may be related to the long period of isolation probably undergone by these people in earlier times, and may not necessarily indicate that the Southern Negro peoples have been in contact with them for extended periods. Its presence in the Natal Negroes therefore, in company with their high A_2 frequency, probably has no significance regarding their origin other than to confirm that they are Southern Negroes.

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WEAK GROUP A VARIANT PHENOTYPES IN THE NATAL INDIANS

4.1 INTRODUCTION

Three different backgrounds have been identified for weak group A variant red cells. They are, rare weak alleles of the *A* gene, expression of normal A antigen on the red cell membrane reduced by the inheritance of dominant or recessive inhibiting or modifying genes, and effects of the environment. The variants known as A_x (Fischer and Hahn, 1935), A_3 (Friedenreich, 1936) and A_m (described but not named as such by Gammelgaard in 1942), are all believed to be due to the inheritance of different weak *A* alleles. The phenotypes A_{end} (Weiner, Sanger and Race, 1959), A_{el} (Reed and Moore, 1964) and A_{bantu} (Brain, 1966) are further examples. However, the A_m-like cells now known as A_y (Weiner, Lewis, Moores, Sanger and Race, 1957) are thought to be due to suppression of a normal A_1 gene by the inheritance of *yy* recessive inhibitor or modifying genes, and those described by Rubinstein, Allen and Rosenfield (1975) to similar suppression by a dominant inhibitor gene. The environment is believed to underlie the mixture of A and weak A cells sometimes seen in advanced age and the weak A cells associated with leukaemia.

The reactions given by weak group A variant red cells vary from weak or negative with anti-A and 1+ positive with anti-A,B reagents to negative with both these reagents. A_{bantu} cells react perhaps the most strongly with anti-A, their mixed-field agglutination with this but not with anti-A,B reagents distinguishing them from A_3 and the weaker phenotype A_{end} cells which are agglutinated in mixed-field patterns both by anti-A and anti-A,B reagents. A_x cells, which are not agglutinated by anti-A, are agglutinated in normal rather than in mixed-field patterns by anti-A,B reagents, and A_m, A_{el}, A_y and the A_m-like cells reported by Rubinstein *et al.* (1975) are not agglutinated by either reagent. The four latter cells are distinguished from one another both by the ease with which anti-A may be eluted from them after they have been exposed to this antibody and by the amount of A substance detected in the saliva of the secretors. A_m cells elute anti-A readily, A_{el} and A_m-like cells elute it less readily, and A_y cells, from the first known examples of which no anti-A was eluted, may elute traces of anti-A (Cartron, Gerbal, Badet, Ropars and Salmon, 1975; Salmon, 1976). The saliva of the people who have A_m cells and who are

secretors contains plenty of A substance, the saliva of those who have A_y and A_m-like cells contains less A substance, and no A substance is detected in the saliva of those who have A_{el} cells.

Anti-A_1, but not anti-A, allo-antibodies are often detected in the serum of persons who have A_{bantu}, A_x and A_{el} cells, are detected occasionally in those who have A_{end} cells and are usually not detected in those who have A_m, A_m-like and A_y cells. The serum of those who have A_m cells also contains only 30–50% of the α-2-N-acetylgalactosaminyltransferase expected when the cell phenotype is A_1 or A_2 (Cartron *et al.*, 1975). This enzyme has not been detected in the serum of persons who have A_y cells.

The frequency of the A_x phenotype was assessed by Salmon (1960) as being 1 in 40 000 of French blood donors, and only a few families and individuals who have A_m, A_{end}, A_{el} or A_y cells have been recorded (Race and Sanger, 1975). A_m-like cells (the result of dominant suppression by an A_1 gene) appear to have been described only once, but A_{bantu} cells occur in approximately 4% of group A Natal Blacks (Brain, 1966). The relatively high frequency of the A_{bantu} phenotype in Natal has unfortunately led to weak group A variant cells from Blacks, Whites and Indians being seldom referred for further studies, and this is consequently the reason so few Indians with these cells have been included in my thesis. The frequency of weak group A variant phenotypes, taken as a whole but excluding A_2, in India was recorded by Bhatia and Sathe (1974) as being 1 in 6 000 of group A Indians (twice as many with A_x as with A_m variant cells were detected in their series), or 1 in 16 700 of the Indian population.

In this chapter, four Natal Indians with weak group A variant red cells will be described. The findings showed that the cells of two of the Indians were A_x and of two A_m-like. As the families of the two Indians who had A_m-like cells were not informative, the results obtained with their cells were compared with those of the individuals with A_m and A_m-like cells recorded elsewhere (Race and Sanger, 1975) and their cells were identified provisionally as A_y.

4.2 CASE HISTORIES

The two Natal Indians who had A_x cells in this study were Mrs K. Cas., a 24 year old Moslem who was three months pregnant, and Mrs Poon., a Tamil-speaker who was

also pregnant. Both women were in good health and were discovered when samples of their blood were received by the laboratory for routine antenatal tests. The two Natal Indians who had A_m-like cells (A_y) were Mrs S. Nai., who spoke Telegu, was pregnant for the second time and was discovered in the same way, and G. Pir., a healthy, 21 year old, Hindi-speaking blood donor.

4.3 MATERIALS AND METHODS

The samples used in this study were clotted blood, serum separated immediately the blood had been received, and saliva. The preparation of these samples for use has been described in Chapter 2. The ABO reagents used were standardised anti-A, anti-anti-B and anti-A,B, six high-titre human immune anti-A, 20 sera containing anti-A,B from White and four from Black antenatal patients who were selected at random, six sera containing low titre anti-A,B from unstimulated White male blood donors, two high-titre rabbit immune anti-A previously absorbed for unwanted agglutinins, anti-A_{HP} from a snail (kindly supplied by Dr M.C. Botha, Provincial Blood Grouping Laboratory, Cape Town) and *Lima* bean anti-A lectin. Anti-A+B+H from a Natal O_h Indian donor, two naturally-occuring anti-H from group A_1 donors, anti-I and anti-i from known donors, *Ulex* anti-H lectin and *Arachis hypogea* anti-T lectin were also used. The eluates were made either by the 56°C heat technique of Landsteiner and Miller (1925) or by the ether technique of Vos and Kelsall (1956) in saline from cells sensitized with human immune anti-A. These techniques are also described in Chapter 2. The tests were made by the saline, one-stage 0,5% bromelin and one-stage 0,25% ficin tile techniques at 22°C and at 4°C, and the eluates were tested by the indirect saline antiglobulin technique as well, using a broad-spectrum antiglobulin reagent obtained from a commercial source.

4.4 RESULTS

4.4.1 First two Natal Indians with weak A variant red cells

The cells of Mrs K. Cas. and Mrs Poon. were not agglutinated by standardised anti-A, anti-B or snail anti-A_{HP}, but they were agglutinated moderately strongly by commercial anti-A,B and microscopically by high titre human and rabbit immune anti-A sera (Table 4.1). Their cells were agglutinated normally in these tests, and not in the mixed-field pattern associated with A_3 and A_{end} cells tested with anti-A and anti-A,B reagents. Their cells were also not agglutinated in the mixed-field pattern

seen with A_{bantu} cells tested in parallel with anti-A. Neither the strength nor the number of the cells agglutinated was improved by incubating the tests at 4°C instead of at 20°C, and anti-B but no anti-A or anti-A_1 allo-antibodies were identified in their sera. It should perhaps be re-stated here that anti-A_1 is detected regularly in the serum of the Natal Blacks who have phenotype A_{bantu} cells.

TABLE 4.1

Results of tests with phenotype A_x cells and sera of two Natal Indians and with phenotype A_{bantu} cells and serum of a Natal Black

Cells and serum	Group	Anti-A	Anti-B	Anti-A,B	A_1 cells	A_2 cells	B cells	O cells
Mrs K. Cas.	A_x	–	–	1	–	–	4	–
Mrs Poon.	A_x	–	–	1	–	–	4	–
Natal Black	A_{bantu}	(2)$^{\cdot}$	–	1$^{\cdot}$	1	–	4	–
Control	Positive	4	4	4				
Control	Negative	–	–	–				

Key: (2)$^{\cdot}$, 1$^{\cdot}$ = mixed-field agglutination.

The I and i antigen strength of the cells of Mrs K. Cas. and Mrs Poon. appeared normal and their cells were not T-sensitized. Since it was evident that their cells were group A, no absorption-elution tests with anti-A were made. In their case, no saliva samples were received and, unfortunately, the families of the Indians were not available for family inheritance studies.

4.4.2 Second two Natal Indians with weak A variant red cells

The cells of Mrs S. Nai. and G. Pir. were not agglutinated by anti-A, anti-B or anti-A,B in normal tests or when nine volumes of these reagents were added to one volume of cell suspension (Table 4.2, part a). Their cells were also not agglutinated by 20 anti-A,B selected at random from White and four from Black antenatal patients, in some of whom this antibody was expected to be undergoing current stimulation by

TABLE 4.2

Results of tests with A_m-like cells and sera of two Natal Indians

Part a

		anti-A			anti-B			anti-A,B			anti-A+B+H
		Saline	Bromelin	Ficin	Saline	Bromelin	Ficin	Saline	Bromelin	Ficin	Saline
Mrs S. Nai.	A_m-like	–	–	–	–	–	–	–	–	–	4
G. Pir.	A_m-like	–			–			–			4
Control	Positive	4	4	4	4	4	4	4	4	4	4
Control	Negative	–	–	–	–	–	–	–	–	–	–

Part b

		A_1 cells			A_2 cells			B cells			O cells		
		Saline	Bromelin	Ficin	Saline	Bromelin	Ficin	Saline	Bromelin	Ficin	Saline	Bromelin	Ficin
Mrs S. Nai.	(1)	1	2	–	–	–	4	4	4	–	–	–
G. Pir.	–			–			4			–			

an ABO incompatible fetus, by high-titre human and rabbit immune anti-A, by snail anti-A_{HP}, by *Lima* bean anti-A lectin or by low titre anti-A,B in the sera of six unstimulated male blood donors. The male donors' sera were included as they were more likely to contain a higher proportion of type IgM anti-A,B than the sera of stimulated donors. However, the cells of Mrs S. Nai. and G. Pir. were agglutinated by anti-A+B+H, by both examples of naturally-occurring anti-H from group A_1 donors and by *Ulex* anti-H lectin. In subsequent titrations with the *Ulex* anti-H, Table 4.3 shows that their cells were agglutinated to the same titre as the control O cells. The strength of the I and i antigens of their cells appeared normal and their cells were not T-sensitized.

TABLE 4.3

Ulex anti-H titration results with phenotype A_m-like cells of two Natal Indians

Cells	Group	*Ulex* anti-H Dilutions in saline						
		1	2	4	8	16	32	64
Mrs S. Nai.	A_m-like	4	4	4	4	3	1	–
G. Pir.	A_m-like	4	4	4	3	2	1	–
Control	A_1	1	(2)	–	–	–	–	–
Control	A_2	4	4	3	2	1	–	–
Control	B	2	1	–	–	–	–	–
Control	O	4	4	4	4	3	1	–

The eluates recovered from the cells of Mrs S. Nai., using the 56°C heat technique, and from the cells of G. Pir., using the ether technique, after their cells had been exposed to human immune anti-A, failed to agglutinate A_1 and A_2 cells in saline tests at 20°C. However, their eluates did agglutinate these cells, and not O cells, microscopically in one-stage 0,5% bromelin tests at this temperature. The weak reactions of their eluates was in marked contrast to the strong reactions obtained when similar eluates were recovered instead from A_{bantu} control cells and tested in the same way.

TABLE 4.4

Results of inhibition tests with saliva of Natal Indian with A_m-like cells

Saliva	Group	Anti-A and A_1 cells Dilutions in saline									
		1	2	4	8	16	32	64	128	256	512
Mrs S. Nai.	A_m-like	4	4	4	4	3	2	1	–	–	–
Secretor	O	4	4	4	4	4	3	2	1	(2)	–
Secretor	A	(1)	–	–	–	–	–	–	–	–	–
Non-secretor	A	4	4	4	4	3	3	3	1	(2)	–
Saline		4	4	4	4	3	3	2	1	(3)	–

Saliva	Group	Anti-B and B cells Dilutions in saline									
		1	2	4	8	16	32	64	128	256	512
Mrs S. Nai.	A_m-like	4	4	4	4	4	3	3	1	(2)	–
Secretor	O	4	4	4	4	4	3	2	1	(2)	–
Secretor	A	–	–	–	–	–	–	–	–	–	–
Non-secretor	A	4	4	4	4	4	3	2	1	(2)	–
Saline		4	4	4	4	4	3	2	1	(3)	–

Saliva	Group	Anti-H *Ulex* and O cells Dilutions in saline									
		1	2	4	8	16	32	64	128	256	512
Mrs S. Nai.	A_m-like	–	–	–	–	–	–	–	–	–	–
Secretor	O	–	–	–	–	–	–	–	–	–	–
Secretor	A	–	–	–	–	–	–	–	–	–	–
Non-secretor	A	4	4	2	1	(1)	–	–	–	–	–
Saline		4	4	3	2	1	–	–	–	–	–

Saliva inhibition tests confirmed that G. Pir., whose red cells typed as Le(a+b–), was a non-secretor of ABH substances. The saliva of Mrs S. Nai., who had Le(a–b+) red cells, inhibited anti-H strongly and anti-A weakly (Table 4.4). These results showed that Mrs S. Nai. was a secretor of H and of a small quantity of A substances.

In the serum of G. Pir., anti-B but no anti-A or anti-A_1 allo-antibodies were detected. However, the serum of Mrs S. Nai. contained anti-B and weak anti-A_1 (Table 4.2, part b).

The three-years old first child of Mrs S. Nai. was the only member of her family tested. Her cells were group O and her serum contained anti-A,B allo-antibodies of normal strength. Saliva inhibition tests showed that she was a non-secretor of ABH substances. The family of G. Pir. was unfortunately not available for study.

4.5 DISCUSSION

The cells of the first two Indians described in this study, Mrs K. Cas. and Mrs Poon., were identified confidently as phenotype A_x as their cells were not agglutinated by anti-A and were agglutinated in normal, rather than in mixed-field, patterns by anti-anti-A,B reagents. Although no anti-A_1 allo-antibodies were detected in their sera, this was not of serious concern as anti-A_1 is not detected invariably in A_x persons. Moreover, its absence in Mrs K. Cas. and Mrs Poon. leant support to the view that their cells were not phenotype A_{bantu}, the weak group A variant phenotype found in 4% Natal group A Blacks. A_{bantu} may also be distinguished from A_x cells by the former being agglutinated by anti-A in mixed-field patterns.

The cells of the second two Indians described in this study, Mrs S. Nai. and G. Pir., clearly had very little A antigen expression. As their cells were not agglutinated by anti-A or by anti-A,B reagents, the phenotype of their cells was either A_m, A_{el}, A_y or A_m-like. Since anti-A had been eluted with difficulty from the cells of both Indians and the saliva of Mrs S. Nai. contained a minimal amount of A substance, the most likely phenotype of their cells was A_y, but the serum of Mrs S. Nai. contained weak anti-A_1 allo-antibodies. The possibility that the Indians had inherited *yy* inhibitor genes which had suppressed the expression of normal A_1 genes in them had also not been confirmed through adequate family studies. As a result, the phenotype of their cells was identified as A_y with reserve (Table 4.5).

TABLE 4.5

Results reported for persons with weak group A variant cells elsewhere and those found with two Natal Indians whose cells were A_m-like

Phenotype	Reactions with anti-A	Reactions with anti-A,B	Strength of anti-A in eluate	Strength of A substance in saliva	Strength of anti-A_1 in serum
A_{bantu}	(2)$^{\bullet}$	+$^{\bullet}$	strong	no	yes
A_3	+$^{\bullet}$	+$^{\bullet}$	strong	yes	sometimes
A_{end}	(1)$^{\bullet}$	(2)$^{\bullet}$	strong	no	sometimes
A_x	–	+	strong	usually no	yes
A_m	–	–	strong	strong	no
A_y	–	–	moderate	weak	no
A_{el}	–	–	intermediate	no	yes
Dominant inhib. of A	–	–	weak	weak	no
G. Pir.	–	–	weak	non-secretor	no
Mrs S. Nai.	–	–	weak	weak	yes

Key: +$^{\bullet}$ = mixed-field agglutination.

No previous accounts of Indians from Natal with weak group A variant red cells were found.

4.6 SUMMARY

Four Natal Indians with weak group A variant red cells, the cells of two of whom were identified as phenotype A_x and the other two provisionally as phenotype A_y, established that weak A variant cells other than A_{bantu} were present in Natal.

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Paper 25

Weak B

2. *No anti-B in serum (or very weak cold anti-B); B in saliva*

Levine, Celano and Griset[65]: a dominant character, called B_w, found in several members of a negro family. Yokoyama, Stacey and Dunsford[66,176]: a dominant character, called B_x, in three members of a Japanese family. Yokoyama, Barber and Dunsford[186]: a dominant character in a Japanese family. Liotta, Russo and Gandini[330]: in a Roman donor with no available relatives. Kout and Totin[143]: one member of a Prague family, considered B_x. Yamaguchi, Okubo, Hazama and Oyama[144]: three Japanese families, two of them showing the character to be dominant; the notation B_x was used. Furukawa and Iseki[145]: a dominant character in a Japanese family; notation B_m. Ikemoto, Kuniyuki and Furuhata[231]: a large Japanese family also illustrating dominance; notation B_m. Simmons and Kwa[489]: five members of two Chinese families. Sathe *et al.*[537]: seven members of a Bombay family also illustrating dominance; notation B_m. Garlick and Maldre[538]: two Canadian sisters; notation B_m. Moores[539]: eleven members of an Indian kindred. Ikemoto *et al.*[540,541]: thirteen members of a Japanese kindred; notation B_m. Kogure and Iseki[542]: a three generation Japanese family with some disturbance of the normal inheritance; notation B_m.

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Study 3

WEAK GROUP B VARIANT PHENOTYPES IN THE NATAL INDIANS

5.1 INTRODUCTION

Variants of blood group B first began appearing in the literature in 1955. In general their characteristics are: weak or no agglutination of the red cells by anti-B and Anti-A,B reagents, H antigen usually expressed strongly, anti-B usually easily eluted after their exposure to this reagent, no or at the most weak anti-B in the serum and B and H, but sometimes only H, substances present in the saliva of secretors. Some examples were considered to be due to the inheritance of an allele of the *B* gene (Sathe, Sharma, Bhatia and Sahiar, 1966), some to reduction of normal *B* gene expression on the membranes of the cells by an inhibiting or modifying gene which might be a linked (Bhatia, Undevia and Sanghvi, 1965) or an inherited (Gundolf and Anderson, 1970; Marsh, Ferrari, Nichols, Fernandez and Cooper, 1973) mutant regulator, some to a B-like antigen "acquired" by the cells as the result of action by certain bacteria (Cameron, Graham, Dunsford, Sickles, Macpherson, Cahan, Sanger and Race, 1959) and at least one to leukaemia (Undevia, Bhatia, Sharma and Parekh, 1966). The variant B_m is believed (Watkins, 1978) to be caused by an inhibitor gene which results in a genetic block at the level of B antigen expression on the red cell membrane as both B and H transferases are present in the serum, and the variant B_3 examples so far recorded appear to be heterogenous (Lopez, Bouguerra, Lemeud, Badet and Salmon, 1974).

Although the group B phenotype frequency is more than 30% in most Indian populations and it nearly always exceeds the Indian group A phenotype frequency (± 20%; Mourant, Kopeć and Domaniewska-Sobczak, 1976), the number of Indian individuals and families with weak group B variant red cells reported from India is almost three times lower than the number reported with weak group A variant red cells (Bhatia and Sathe, 1974). Bhatia and Sathe (1974) gave the weak group B variant phenotype frequency in India as being 1 in 9 300 group B Indians or 1 in 24 000 Indians of all ABO groups. Four of the 11 weak group B variant Indians in their series were Moslems, two were Christians, four were Hindus and the religion of one was not known. As these studies were made in the Indian States of Maharashtra and Gujarat, the language spoken by the non-Moslems is likely to have been Hindi. By contrast, 32 Indians with weak group A variant red cells were recorded in the same series.

In Natal, three Indian blood donors whose red cells were shown in my tests to be weak group B variant were found among 67 000 Indians who donated their first units of blood at Natal Blood Transfusion Service donor clinics in the years between 1965 and 1980. The likelihood that other donors with this phenotype were missed was low as it had always been customary to refer all donations giving unusual results for further tests. The frequency of this phenotype is therefore approximately 1 in 7 560 group B Natal Indian blood donors or 1 in 22 680 Natal Indian blood donors of all ABO groups, and it does not differ significantly ($P = < 0,98$) from the frequency reported for Indians in India. In addition, five further Natal Indians with weak group B variant red cells were investigated, supported wherever possible by family studies. The apparent scarcity of Indians with weak group B variant phenotypes is strange in view of the finding by Milner and Calitz (1968) that the strength of the B antigen on the red cells of normal group B Natal Indians varies considerably. In a quantitative study, the cells of some of the group B Natal Indians tested by these investigators were agglutinated more strongly by anti-B than the strongest-agglutinated group B Zulu cells tested (the cells of the Zulus often have extra-strong B antigen; Brain, 1966) while the cells of other group B Indians tested were agglutinated more weakly than the most weakly-agglutinated group B White cells tested.

The weak group B variant red cells which react weakly or not at all with anti-B and anti-A,B were sub-divided by Race and Sanger (1962) into three categories. In *Category 1* were placed those cells, called B_v, in the sera of the possessors of which anti-B was present and in the saliva of the secretors a kind of B substance in addition to H substance had been detected. In *Category 2*, the cells, usually called B_m, were from persons who had no or almost no anti-B in their sera and the secretors secreted both B and H substance in their saliva; and in *Category 3*, the cells, called B_3 or B_x, but not having quite the same characteristics as A_3 and A_x cells, belonged to persons who had no anti-B in their sera. The secretors in *Category 3* had H but no B substance in their saliva. Salmon (1976), however, who believes that the terminology should correspond as far as possible with the terminology used for weak group A variant red cells, recently suggested retaining the symbol B_3 for the cells which show 'mixed field' agglutination, consisting of small agglutinates among many unagglutinated cells in the presence of anti-B and anti-A,B, and using the symbol B_x for the cells which are not agglutinated by anti-B but are agglutinated normally although weakly by anti-A,B. According to Salmon (1976), B_3 secretors have B and H substances in their saliva and B_x secretors a B-like substance which inhibits the agglutination of their cells by the anti-B recovered, after adsorption, from their cells in eluates. The serum of B_x secretors also contains weak anti-B.

The first Indians in India with weak group B, *Category 3*, red cells were recorded by Vyas, Bhatia and Sanghvi in 1960; and the first Indians in India with weak group B, *Category 2* or phenotype B_m red cells by Sathe, Sharma, Bhatia and Sahiar in 1966. In 1974, Bhatia and Sathe reported that the serological reactions of the cells of all the weak group B variant members in some Indian families in India tested with anti-B and anti-A,B were similar, while in other Indian families there the cells of some of the weak group B variant members reacted with these reagents differently from others. In this chapter, the serological findings in the first two Indian families in Natal with *Category 2* or phenotype B_m red cells will be described. The families are believed to be only the second and third with this phenotype to be recorded in Indians, and the cells of all the 11 phenotype B_m members in the first family reacted similarly in the appropriate tests. Four other Natal Indian individuals whose cells and sera reacted in the same manner as those of the weak group B variant individuals in these two families and whose cells were called B_m-like as family studies were not made, and two Natal Indians whose cells were called *Category 3* – or phenotype B_3-like, will also be described.

5.2 CASE HISTORIES

An. Gov, the propositus in the first Natal Indian family with *Category 2* or phenotype B_m red cells, was 40 years of age, in good health and spoke Tamil. He was discovered when his first blood unit, donated at an N.B.T.S. clinic in 1968, was grouped in manual tests as O, but anti-A only was detected in his serum. An. Mur., the propositus in the second Natal *Category 2* Indian family, and M. Kri., a Natal Indian individual with *Category 3* – or phenotype B_3-like cells, both of whom were also healthy blood donors and who spoke Tamil, were detected in the same way in 1979 and 1978 respectively. The two Natal *Category 2* or phenotype B_m Indian women, Mrs Sob., aged 24 years and Mrs Sta., aged 16 years; and the second Natal Indian individual, with *Category 3* – or phenotype B_3-like cells, Mrs S. Pil., aged 19 years; all of whom spoke Tamil and who were pregnant, were identified when routine blood samples were submitted from them to the laboratory for antenatal tests. The two other Natal *Category 2* or phenotype B_m Indian individuals, Mrs Ch. and her young son G. Ch., who both spoke Telegu, were participants in a disputed paternity suit whose blood samples were received for confirmation of an apparently weak ABO group.

5.3 MATERIALS AND METHODS

The routine procedure consisted of testing the cells with from 6 to 10 different examples of standardised anti-A, anti-B and anti-A,B reagents, several special high titre immune examples of these antibodies, serial dilutions in saline of *Ulex* anti-H reagent, and AB serum. Tests with lectins specific for various types of red cell polyagglutination were included as well, when these became available. The anti-A, anti-B, anti-A,B and AB serum reagents were used by the saline, one-stage 0,5% bromelin and one-stage 0,25% ficin techniques, whenever possible at 22°C, 10°C and at 4°C, and the lectins were used in accordance with the techniques recommended for each. All these techniques are described in Chapter 2, and suitable positive and negative control cell samples were always included with the tests. The serum from the blood samples was tested by the techniques described in Chapter 2 as well, and the eluates were made in saline by the 56°C technique of Landsteiner and Miller (1925).

5.4 RESULTS

5.4.1 First Natal Indian family with *Category 2* or phenotype B_m red cells (Moores, 1970)

Table 5.1 shows that the red cells of An. Gov., II-4, (Figure 5.1), were not agglutinated in saline, bromelin and ficin tests either at 22°C or at 4°C by the anti-A and anti-B reagents used. However, his cells were agglutinated by the anti-A,B reagents used in saline tests at 4°C and in bromelin and ficin tests at 22°C. In the saline and bromelin tests the agglutination seen was microscopic only, and in the ficin tests weakly macroscopic. Anti-B but not anti-A was readily recovered from his cells in eluates, but the titre of the anti-B reagent absorbed by his cells in these tests was not seen to have been reduced when it was subsequently compared with the titre of the same reagent after absorbtion by group O cells (Table 5.2). The H antigen strength of An. Gov.'s cells was seen to be similar to that of the control group O cells (Table 5.3). A sample of his cells was washed three times, packed down and mixed with an excess of anti-A,B in a Petri dish by intermittent hand rotation at 22°C for one hour, but despite the opportunity provided for improved contact between his cells, they failed to produce macroscopic agglutinates by this technique. Anti-A but not anti-B was detected in An. Gov.'s serum, and his saliva, which inhibited anti-B and anti-H but not anti-A, showed by this that he was a secretor of B and H substances (Table 5.4). The amount of these substances secreted by An. Gov. was found to be similar to the

TABLE 5.1

Results of ABO grouping tests with the cells of An. Gov., phenotype B_m, and his family members

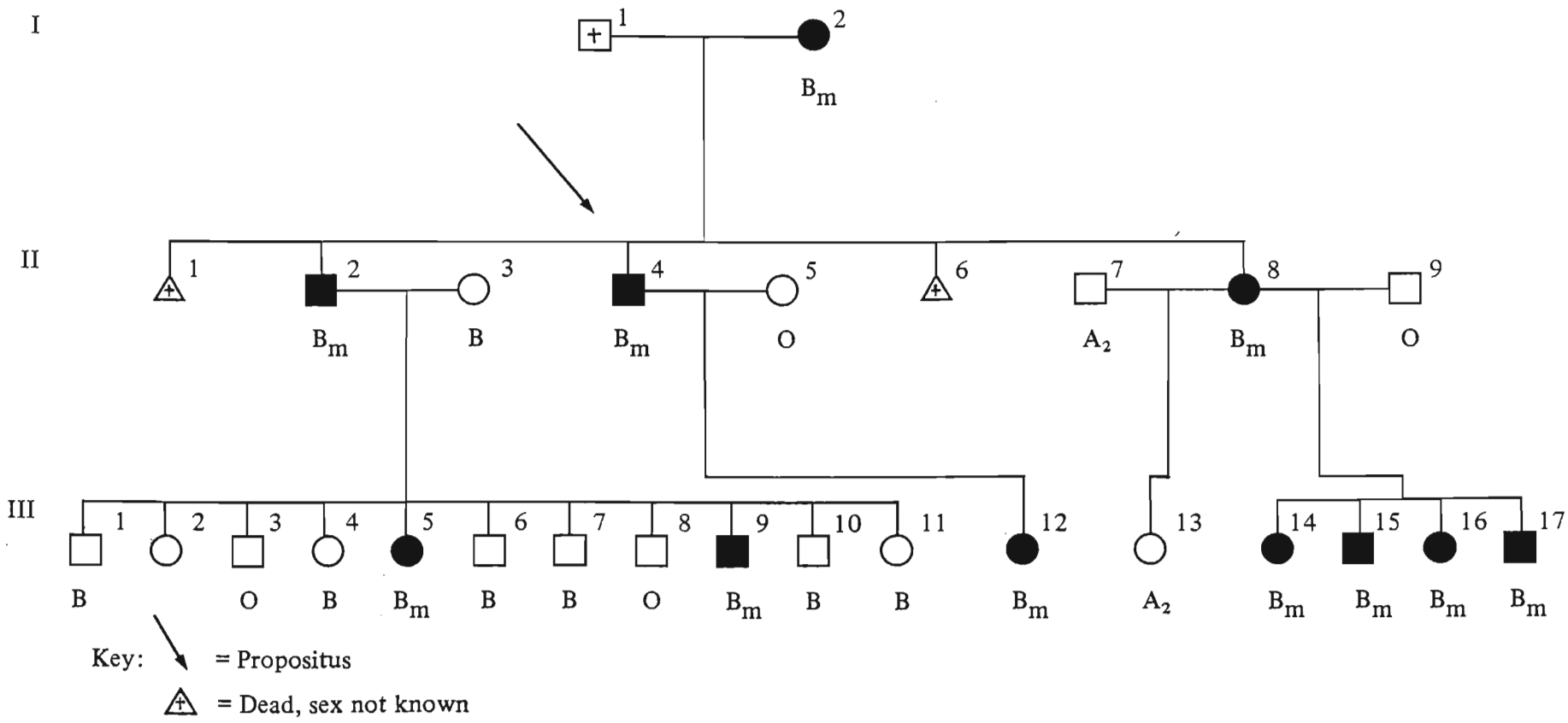
	SALINE						0,5% BROMELIN						0,25% FICIN					
	Anti-A		Anti-B		Anti-A,B		Anti-A		Anti-B		Anti-A,B		Anti-A		Anti-B		Anti-A,B	
	22°	4°	22°	4°	22°	4°	22°	4°	22°	4°	22°	4°	22°	4°	22°	4°	22°	4°
I-2	–	–	–	–	(1)		–	–	–	–	(2)		–	–	–	–	1	
II-2	–	–	–	–	–	(±)	–	–	–	–	(2)		–	–	–	–	1	
II-4 ↙	–	–	–	–	–	(3)	–	–	–	–	(3)		–	–	–	–	2	
II-8	–	–	–	–	–	(1)	–	–	–	–	(2)		–	–	–	–	±	
III-5	–	–	–	–	–	1	–	–	–	–	–		–	–	–	–		1
III-9	–	–	–	–	–		–	–	–	–	–		–	–	–	–		1
III-12	–	–	–	–	–	–	–	–	–	–	1		–	–	–	–	±	
III-14	–	–	–	–	–		–	–	–	–	(2)		–	–	–	–	1	
III-15	–	–	–	–	(±)		–	–	–	–	(3)		–	–	–	–	2	
III-16	–	–	–	–	–		–	–	–	–	(2)		–	–	–	–	1	
III-17	–	–	–	–	(±)		–	–	–	–	(2)		–	–	–	–	2	

Key: 22° = 22° Centigrade

↙ = propositus

FIGURE 5.1

Family of An. Gov.



amount secreted by his normal group B relatives (Table 5.4). These findings, confirmed when further samples were obtained from An. Gov. one year later and recorded as such by Race and Sanger in *Blood Groups in Man* (6th ed. p 20, 1975), established that An. Gov. had *Category 2* or phenotype B_m red cells.

TABLE 5.2

Results of titrating with B cells the anti-B and anti-A,B reagents absorbed with the cells of An. Gov.'s family members

Reagent	Absorbing cells	Dilutions of anti-B 1	2	4	8	16	32	64	128	256	512	1024	Score
Anti-B	II-4 B_m	4	4	4	4	4	4	3	2	2	(2)	–	87
	II-5 O	4	4	4	4	4	4	4	2	1	(1)	–	83
	Unabsorbed	4	4	4	4	4	4	4	4	2	(3)	(1)	90

Reagent	Absorbing cells	Dilutions of anti-A,B 1	2	4	8	16	32	64	128	256	512	1024	Score
Anti-A,B	II-4 Bm	4	4	4	4	4	4	4	2	1	(1)	–	83
	II-5 O	4	4	4	4	4	4	4	2	1	(2)	–	84
	Unabsorbed	4	4	4	4	4	4	4	3	2	(3)	–	90

5.4.1.1 Family study and discussion

Blood and saliva samples from members of An. Gov.'s family disclosed that, in addition to himself, 10 family members had *Category 2* or phenotype B_m red cells (Figure 5.1; Tables 5.1 and 5.5). The reactions of the cells of some of the family members were fractionally weaker with the anti-A,B reagents used than the cells of others (Table 5.1) but the small differences were not considered significant. In titrations with *Ulex* anti-H, the H antigen strength of their cells was seen to be similar to that of the group O family members' and the group O control cells (Table 5.3), and the serum of II-2, like that of II-4, contained anti-A but no anti-B allo-antibodies. The family members who had phenotype B_m cells and who were secretors secreted approximately the same amount of B and H substances as the group B secretor family members (Table 5.4). The group O phenotypes of III-3 and III-8, and the group A_2

TABLE 5.3

Results of *Ulex* anti-H titrations with the cells of An. Gov. and his family

Family number	ABO phenotype	Ulex anti-H titre				
		2	4	8	16	32
I-2	B_m	3	2	2	1	±
II-2	B_m	4	3	2	1	±
II-4	B_m	4	3	2	1	±
II-8	B_m	4	3	2	1	±
III-5	B_m	4	3	2	1	±
III-9	B_m	4	3	2	1	–
III-12	B_m	4	3	2	1	±
III-14	B_m	4	3	2	±	–
III-15	B_m	4	3	2	±	–
III-16	B_m	4	3	2	±	–
III-17	B_m	4	3	2	±	–
III-1	B	2	2	1	–	–
III-4	B	2	2	±	–	–
III-6	B	2	2	1	–	–
III-7	B	2	2	1	–	–
III-10	B	2	2	±	–	–
III-11	B	3	2	1	–	–
II-3	B	3	2	1	–	–
II-9	O	4	3	2	1	±
III-3	O	4	4	3	1	±
III-8	O	4	3	2	1	–
II-5	O	4	3	2	1	±
III-13	A_2	4	2	2	1	–
CONTROL	B	2	2	±	–	–
CONTROL	O	4	3	2	1	±
CONTROL	A_1	1	±	–	–	–

TABLE 5.4

Results of titrations in which saliva from An. Gov. and his family members were used to inhibit anti-B and *Ulex* anti-H reagents

Family No.	Group	Anti-B diluted 1 in 20 Group B cells									Ulex anti-H diluted 1 in 8 Group O cells								
		2	4	8	16	32	64	128	256	512	2	4	8	16	32	64	128	256	512
I-2	B_m		–	–	±	1	2	2	4	4	–	±	1	2	3	4	4	4	4
↘ II-2	B_m	–	–	–	–	–	–	±	1	2	–	–	–	±	1	2	3	4	4
II-4	B_m	–	–	–	–	–	±	1	1	2	–	–	±	2	4	4	4	4	4
II-8	B_m	–	–	–	–	–	–	±	1	1	–	–	±	1	2	3	4	4	4
III-5	B_m	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
III-9	B_m	–	–	–	–	–	–	±	1	1	–	–	±	1	2	3	4	4	4
III-12	B_m	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
III-14	B_m	–	–	–	–	–	±	1	1	2	–	–	±	2	2	3	4	4	4
III-15	B_m	–	–	–	–	–	±	1	1	2	–	–	±	1	1	3	4	4	4
III-16	B_m	–	–	–	–	–	±	1	1	2	–	–	±	1	2	4	4	4	4
III-17	B_m	–									–								
II-3	B	–	–	–	–	–	±	1	1	2	–	–	–	±	1	3	4	4	4
III-1	B	–	–	–	–	–	±	1	1	2	–	±	1	3	4	4	4	4	4
III-4	B	–	–	–	–	–	–	±	1	1	–	–	±	1	2	4	4	4	4
III-6	B	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
III-7	B	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
III-10	B	–	–	–	–	–	–	–	±	1	–	±	1	2	4	4	4	4	4
III-11	B	–									–								
II-5	O	4	4	4	4	4	4	4	4	4	–	–	–	–	±	1	1	2	2
II-9	O	not tested									not tested								
III-3	O	4	4	4	4	4	4	4	4	4	–	–	–	–	–	–	±	1	1
III-8	O	4	4	4	4	4	4	4	4	4	–	–	–	–	±	1	2	2	3
III-13	A_2	not tested									–	–	–	–	–	–	–	±	1
Control	B	–	–	–	–	–	–	–	–	±	–	–	–	–	–	±	1	1	2
Control	O	not tested									–	–	–	–	–	–	–	±	1
Control	Saline	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

Key: ↘ = propositus

TABLE 5.5

Blood groups of An. Gov. and his family members

Family member	Age in years	ABO	Substances in saliva	MNSs	P_1	Rhesus	Lu^a	K	Lewis	Fy^a	I
I-2	60	B_m	B.H.	NS/Ns	–	CDe/CDe	–	–	Le(a–b+)	+	+
II-2	45	B_m	B.H.	Ms/NS	–	CDe/CDe	–	–	Le(a–b+)	+	+
II-3	adult	B	B.H.	Ms/Ns	+	cDE/cde	–	–	Le(a–b+)	+	+
II-4 ↙	40	B_m	B.H.	MNS	–	CDe/CDe	–	–	Le(a–b+)	+	+
II-5	adult	O	H	MNS	–	CDe/CDe	–	–	Le(a–b+)	+	+
II-8	38	B_m	B.H.	Ms/Ns	+	CDe/CDe	–	–	Le(a–b+)	+	+
II-9	adult	O	not tested	MS/NS	+	CDe/cde	–	–	Le(a–b+)	+	+
III-1	22	B	B.H.	Ms/NS	+	CDe/cDE	–	–	Le(a–b+)	+	+
III-3	20	O	H	Ms/NS	+	CDe/cDE	–	–	Le(a–b+)	+	+
III-4	16	B	B.H.	Ms/Ms	+	CDe/cde	–	–	Le(a–b+)	+	+
III-5	14	B_m	–	Ms/NS	+	CDe/cde	–	–	Le(a+b–)	+	+
III-6	13	B	–	NS/NS	+	CDe/cDE	–	–	Le(a+b–)	+	+
III-7	11	B	–	NS/Ns	+	CDe/cde	–	–	Le(a+b–)	+	+
III-8	8	O	H	Ms/Ns	–	CDe/cDE	–	–	Le(a–b+)	+	+
III-9	7	B_m	B.H.	NS/Ns	+	CDe/cde	–	–	Le(a–b+)	+	+
III-10	6	B	B.H.	Ms/Ns	+	CDe/cDE	–	–	Le(a–b+)	+	+
III-11	4	B	B.H.	Ms/NS	+	CDe/cde	–	–	Le(a–b+)	+	+
III-12	9	B_m	–	N.NS	–	CDe/CDe	–	–	Le(a+b–)	+	+
III-13	16	A_2	A.H.	Ms/Ms	+	CDe/cde	–	–	Le(a–b+)	+	+
III-14	13	B_m	B.H.	MNS	+	CDe/cde	–	–	Le(a–b+)	+	+
III-15	11	B_m	B.H.	NS/Ns	+	CDe/CDe	–	–	Le(a–b+)	+	+
III-16	9	B_m	B.H.	MNS	+	CDe/cde	–	–	Le(a–b+)	+	+
III-17	5	B_m	B.H.	MS/Ms	+	CDe/CDe	–	–	Le(a–b+)	+	+

Key: ↙ = propositus

phenotype of III-13 (Figure 5.1) showed by this that II-2 and II-8 were heterozygous B_m/O, and the family pedigree showed that the genes responsible for the B_m phenotype were being inherited in this family in an apparently straight-forward manner. As none of the children of II-4 and II-3 unexpectedly had normal group B cells, it seemed possible, in this family, either that the expression of a normal *B* gene was being affected by linked mutant regulator gene or that a true allele, B_m, of the *B* gene was being inherited.

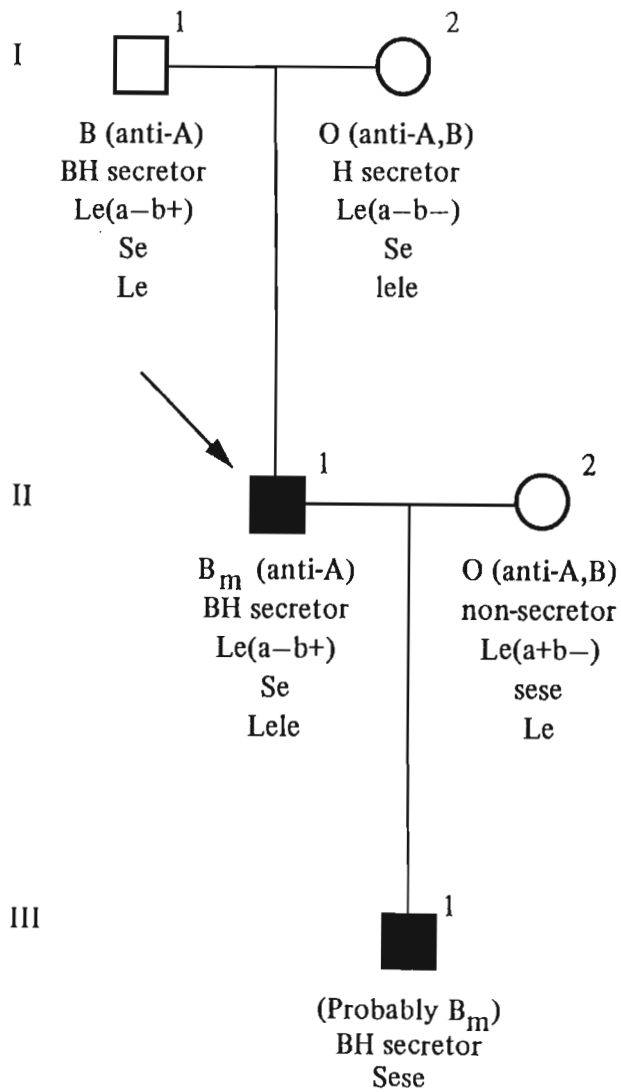
5.4.2 Second Natal Indian family with *Category 2* or phenotype B_m red cells

Unlike the red cells of An. Gov. and members of his family, the cells of An. Mun., II-1, (Figure 5.2) the propositus in the second family with the B_m phenotype, which were not agglutinated by anti-A and anti-B, were agglutinated by the anti-A,B reagents used only in one stage 0,25% ficin tile tests. However, the weaker results obtained with his cells were considered either to be due to a minor quantitative variation of B antigen strength in them or to fewer B antigen sites being available, rather than to a B_m sub-type. The H antigen strength of the cells of II-1 was found to be similar to that of the group O control cells (Tables 5.6 and 5.7) and anti-B but not anti-A was readily eluted from his cells after they had been exposed to these two reagents. Like the anti-B reagent absorbed by An. Gov.'s cells, the anti-B reagent absorbed by the cells of II-1 was shown subsequently not to have undergone a reduction in titre. Anti-A and a cold agglutinin of undetermined specificity, but not anti-B, were detected in the serum of II-1 (Table 5.6), and B and H substances were detected in his saliva (Table 5.8).

At birth, the red cells of III-1 (Figure 5.2) were found to group as O, but his saliva showed, by inhibiting anti-B and anti-H and not anti-A, that he was a secretor of B and H substances. As the ABO antigens are known to be weaker in the newborn than later in life, the normal B antigen is readily detected on cord blood cells and the ABH substances are well-developed in the saliva (Race and Sanger, p 38 and 314, 1975), the most likely phenotype of III-1 was B_m. The corresponding genes had almost certainly been inherited by III-1 from his father, and the non-agglutination of his cells by anti-A,B, unlike those of his father, was not considered significant at this early stage in his development.

Figure 5.2

Family of An. Mun.



Key: ↘ = Propositus

TABLE 5.6

Results of tests with the *Category 2* or phenotype B_m cells and sera of An. Mun. and B_m-like cells of four other Natal Indians

		CELLS									SERUM												Ulex	Anti-B
		Anti-A			Anti-B			Anti-A,B			A_1 cells			A_2 cells			B cells			O cells			anti-H	recovered
		22°	10°	4°	22°	10°	4°	22°	10°	4°	22°	10°	4°	22°	10°	4°	22°	10°	4°	22°	10°	4°	titre	in eluate
	Saline	–	–	–	–	–	–	–	–	–	4	4	4	4	4	4	–	–	–	–	–	–		
An. Mun.	Bromelin	–	–	–	–	–	–	–	–	–	4	4	4	4	4	4	–	(2)	1	–	(1)	(3)	32	yes
	Ficin	–	–	–	–	–	–	1	2	3	4	4	4	4	4	4	–	1	2	–	1	2		
	Saline	–			–			–			4			2			–			–				
Mrs Sob.	Bromelin	–			–			(2)																yes
	Ficin	–			–			–																
	Saline	–			–			–			4			4			–			–				
Mrs Sta.	Bromelin	–			–			–															32	yes
	Ficin	–			–			–			4						–			–				
	Saline	–			–			(±)			4			4			–			–				
Mrs Ch.	Bromelin	–			(±)			(2)			4			4			–			–			32	
	Ficin	–			–			–			4			4			–			–				
	Saline	–			–			–																
G. Ch.	Bromelin	–			–			1															32	
	Ficin	–			–			–																
Control B	Saline	–			4			4			4			4			–			–			4	yes
Control O	Saline	–			–			–			4			4			4			–			32	no

Key: 22° = 22° centigrade

Bromelin = one-stage 0,5% bromelin tube technique

Ficin = one-stage 0,25% ficin tile technique

5.4.2.1 Family study and discussion

I-1, the father of An. Mun., II-1, was group B with anti-A in his serum and I-2, his mother, group O with anti-A,B in her serum (Figure 5.2). As the other blood group systems studied revealed no evidence of illegitimacy in the family, the most likely genotype of I-1 was B/B_m. In titrations with *Ulex* anti-H, the H antigen of the cells of I-1 was found to be increased in strength in relation to that of the control group B cells (Table 5.7) and inhibition tests showed that his saliva contained less H substance than the saliva of the control group AB secretor. The apparent B_m phenotype of III-1 also provided support for the genotype proposed for I-1, for B_m appeared, in this family (and in the family of An. Gov.), to be due to the inheritance either of a true B_m allele or of a mutant regulator gene linked to the *B* gene. The increased H cell antigen of I-1 was therefore of interest in that it suggested that his ABO genotype was unusual. However, in addition to the wide variation of B red cell antigen strength in the Natal Indians reported by Milner and Calitz (1968), the H red cell antigen strength in them was shown in this thesis (Chapter 1) to vary widely. The quantity of the ABH substances secreted is also known to vary for reasons unconnected with the ABO groups, such as the subject's age. As a result, and in accordance with the well-known observation that group A and group B cell homo- and heterozygosity cannot be judged from the results of serological tests, the true genotype of I-1 was not considered to have been established by this means.

TABLE 5.7

Results of *Ulex* anti-H titrations with the cells of An. Mun. and members of his family which showed that the cells of his father, I-1, had increased H antigen

Red cells	Phenotype	Ulex anti-H 1	2	4	8	16	32	64	Score
I-1	B	4	3	1	(3)	(2)	(1)	–	28
I-2	O	4	4	4	4	4	2	–	58
II-1	B_m	4	4	4	3	1	(1)	–	45
II-2	O	4	4	4	3	2	1	–	53
Control	A_1	1	(2)	–	–	–	–	–	6
Control	A_2	4	4	3	2	1	–	–	43
Control	B	2	1	(2)	–	–	–	–	14
Control	O	4	4	4	3	1	(1)	–	45

TABLE 5.8

Results of titration-inhibition tests with anti-A, anti-B and *Ulex* anti-H reagents and the saliva of An. Mun. and his family members in which the saliva of his father, I-1, showed reduced inhibition of anti-H

Saliva sample	Blood group phenotype	A_2 cells Dilutions of anti-A						B cells Dilutions of anti-B						O cells Dilutions of Ulex anti-H					
		4	8	16	32	64	128	4	8	16	32	64	128	4	8	16	32	64	128
I-1	B	4	2	1	1	(2)	–	–	–	–	–	–	–	2	1	–	–	–	–
I-2	O	4	2	1	(3)	(2)	–	4	4	4	3	2	2	–	–	–	–	–	–
II-1	B_m	4	2	1	1	(2)	–	–	–	–	–	–	–	–	–	–	–	–	–
II-2	O	not tested						not tested						not tested					
Control secretor	AB	–	–	–	–	–	–	1	(2)	–	–	–	–	(1)	–	–	–	–	–
Control non-secretor		4	2	1	1	(3)	–	4	4	4	3	2	2	4	3	2	1	–	–
Control saline		4	3	3	2	1	–	4	4	4	3	2	2	4	4	2	1	–	–

5.4.3 Four Natal Indian invididuals with *Category 2-* or phenotype B_m-like red cells; phenotype study

Table 5.6 shows that the red cells of Mrs Sob., Mrs Sta., Mrs Ch. and G. Ch., like the red cells of An. Gov., the 10 other B_m members of his family and those of An. Mun., were not agglutinated by the anti-A and anti-B reagents used and that they were either not or were only very weakly agglutinated by the anti-A,B reagents used (Table 5.6). Similarly, the strength of the H antigen of the cells of Mrs Sta., Mrs Ch. and G. Ch. resembled that of the control group O cells (Table 5.6). Anti-B was eluted with equal readiness from the cells of Mrs Sob. and Mrs Sta. after they had been exposed to this reagent, and the sera of all except G. Ch., from whom no serum was received, contained anti-A but no evidence of anti-B allo-antibodies. No family studies were made. As the father of G. Ch. was not tested, he and his mother, Mrs Ch., were not regarded as a family for the purposes of this investigation. Table 5.6 also shows that the reactions of the cells of these four Indians were not completely identical in every instance.

5.4.4 Two Natal Indian individuals with *Category 3-* or phenotype B_3-like red cells; phenotype study

The *Category 3-* or B_3-like red cells of the first Natal Indian individual described in this section, M. Kri., were agglutinated both by anti-B and by anti-A,B, but not as strongly as normal group B cells, in saline tests at 22°C. The agglutination was 'mixed field' in type and many unagglutinated cells were present. By contrast, the cells of the second Natal Indian with this phenotype, Mrs S. Pil., were agglutinated in similar tests in 'mixed field' patterns only by the anti-A,B reagents used (Table 5,9).

In titrations, in which the anti-B reagent used was diluted serially in doubling dilutions with 6% bovine albumin in order to avoid the cells in the higher dilutions not being agglutinated because insufficient protein was present, the cells of M. Kri. were observed to agglutinate in 'mixed field' patterns to titre 32 and normal group B control cells to titre 256 (Table 5.10). The H antigen strength of the cells of M. Kri. and of Mrs S. Pil. was seen to be similar to that of the normal group O control cells (Table 5.9 and 5.11). Following their exposure to anti-A and anti-B in separate tests, anti-B but not anti-A was readily recovered from M. Kri.'s cells in eluates, but no attempt was made to separate the agglutinated from the unagglutinated cells first and ascertain whether or not anti-B could be eluted from them subsequently. The titre of the anti-B reagent,

TABLE 5.9

Results of tests with the *Category 3*- or phenotype B_3-like cells and sera of M. Kri. and Mrs S. Pil.

Name	Technique	CELLS									SERUM												Ulex anti-H titre	Anti-B recovered in eluate
		Anti-A			AntiB			Anti-A,B			A_1 cells			A_2 cells			B cells			O cell				
		22°	10°	4°	22°	10°	4°	22°	10°	4°	22°	10°	4°	22°	10°	4°	22°	10°	4°	22°	10°	4°		
M. Kri.	Saline	–			1•			2•			4	4	4	3	4	4	–	–	–	–	–	–		
	Bromelin	–			2•			3•			4	4		3	4		–	–		–	–		32	yes
	Ficin	–			3•			4•			4	4		4	3		–	–		–	–			
Mrs S. Pil.	Saline	–	–		–	–		1•	1•		4			3			–			–				
	Bromelin	–			–			1•															32	
	Ficin	–	–		–	–		±	±		4			4			–			–				
Control B	Saline	–			4			4			4			4			–			–			4	yes
Control O	Saline	–			–			–			4			4			4			–			32	no

Key: 1• = 1+ sized aggutinates amid many unagglutinated cells

22° = 22° centigrade

Bromelin = one-stage 0,5% bromelin tube technique

Ficin = one-stage 0,25% ficin tile technique

TABLE 5.10

Results of anti-B titration with the *Category 3-* or phenotype B_3-like cells of M. Kri.

Cells	Anti-B								
	1	2	4	8	16	32	64	128	256
M. Kri	2•	2•	2•	1•	(3)•	(1)	–	–	–
B	4	4	4	4	4	3	2	1	(3)
O	–	–	–	–	–	–	–	–	–

Key: 2• = 2+ sized agglutinates with some unagglutinated cells present

TABLE 5.11

Results of *Ulex* anti-H titration with the *Category 3-* or phenotype B_3-like cells of M. Kri. and Mrs S. Pil.

Red cells	Phenotype	Ulex anti-H 1	2	4	8	16	32	64	Score
M. Kri.		4	4	4	4	2	1	–	53
Mrs S. Pil.		4	4	4	3	2	±	–	49
Control	A_1	1	±	–	–	–	–	–	6
Control	A_2	4	4	4	3	1	±	–	46
Control	B	3	1	–	–	–	–	–	15
Control	O	4	4	4	4	2	1	–	53

after absorbtion with M. Kri.'s unseparated cells, was found to have been reduced from 1 024 to 64 (Table 5.12), and the serum of M. Kri. and Mrs S. Pil. contained anti-A but no evidence of anti-B (Table 5.9). The saliva of the two Indians could not be examined as no sample was received from Mrs S. Pil. and the Le(a+b–) phenotype of M. Kri.'s cells showed by this that he was almost certainly a non-secretor of ABH substances. Family studies were also not possible as the Indians were both uncooperative.

To exclude the 'mixed field' agglutination of M. Kri.'s cells seen with anti-B and anti-anti-A,B from being due to 'acquired B' antigen as the result of the effect of bacterial action *in vivo* (Race and Sanger, p 31, 1975), his cells were tested with BS I (*Bandeiraea simplicifolia*) lectin. Surprisingly, although normal group B control cells, as expected, were agglutinated strongly, M. Kri.'s cells were not agglutinated by this reagent. However, his serum did not contain the anti-B customarily found when 'acquired B' cells are present and, as 'acquired B' antigen is confined almost entirely to group A cells, the lectin's failure to agglutinate his cells strongly suggested that his B antigen was weaker than usual. Had M. Kri. been a B/O chimaera the proportion of the B cells in whose blood was sufficient to give the same sized agglutinates as those observed in the tests with anti-B, 'mixed fields' of agglutinated and unagglutinated cells would almost certainly have been observed with his cells when tested with this lectin. This was confirmed when the BS I lectin used was seen to agglutinate readily in 'mixed field' patterns artificially-prepared mixtures of from 10% to 50% group B in group O cell suspensions (in parallel tests, the agglutination values 1^{+} and 2^{+} (see Tables 5.9 and 5.10) were obtained in the 10% to 30% mixtures using anti-B). Polyagglutination was excluded by the negative reactions of M. Kri.'s cells with *Arachis hypogea, Dolichos biflorus, Salvia sclarea, Salvia horminum* and *Glycine soja* lectins.

5.4.5 *Category 3*- or phenotype B_3-like red cells; discussion

Like phenotype A_3 cells (Gammelgaard, 1942; Race and Sanger, p 14, 1975), the cells described as B_3 and grouped together in *Category 3* by Race and Sanger (see 5.1, introduction to this chapter) were characteristically agglutinated in 'mixed field' patterns by anti-B and anti-A,B reagents. The agglutinates were small in size and they were greatly outnumbered by unagglutinated cells. In absorbtion-elution tests, B_3 cells absorbed very little anti-B but gave it up readily in eluates and, with difficulty, two apparent cell populations were separated by means of differential agglutination

TABLE 5.12

Results of titrating with B cells the anti-B reagent absorbed with the *Category 3*- or phenotype B_3-like cells of M. Kri.

Reagent	Cells used for absorbtion	Dilutions of anti-B 1	2	4	8	16	32	64	128	256	512	1024	2048	Score
Anti-B	M. Kri.	4	4	3	2	1	(3)	(1)	–	–	–	–	–	45
	unabsorbed	4	4	4	4	4	4	3	2	2	(3)	(2)	–	86

(Sussman, Pretshold and Lacher, 1960) but not by differential centrifugation (Wiener and Cioffi, 1972).

Although Gammelgaard (1942) and Dunsford (1959) had shown that anti-A,B existed which would agglutinate all the cells in A_3 samples and Reed (1964) had successfully eluted anti-A from both the agglutinated and the unagglutinated cells after A_3 blood had been exposed to this antibody, no reports were found of similar attempts having been made to elute anti-B separately from the agglutinated and the unagglutinated cells in B_3 samples (perhaps because complete separation was too difficult to achieve). In all the papers studied, the authors confirmed that the unseparated cells would absorb and elute anti-B, and they then established by means of family studies that their propositi had probably inherited a weak *B* variant allele or mutant regulator gene. As a result, other causes of 'mixed field' agglutination, such as blood transfusion, a bone marrow transplant, a leukaemic change or a chimaera whose blood was a mixture of group B and group O cells were not excluded. From the group O cells (naturally not agglutinated by anti-B) in such a chimaera's blood, this antibody would not have been expected to have been recovered again in eluates. Provided the B_3 is the analogue of the A_3 phenotype, however, anti-B should be recovered from the cells not agglutinated by anti-B in B_3 blood samples.

Both M. Kri. and Mrs S. Pil. were healthy and no history of recent blood transfusions or bone marrow transplants was obtained. A fetal bleed was excluded in Mrs S. Pil.'s case as the proportion of group O cells which would have had to be supposed in her blood was too great to have been derived in this way. The possibility that she was group O with fetal group B cells circulating was excluded as well for the B cells would almost certainly have been haemolysed by the anti-A,B expected in a group O person, and no evidence of this antibody had been detected in her serum.

5.5 DISCUSSION

The findings in the healthy Indians with *Category 2* red cells in the two families described in this chapter established that the genes or alleles responsible for the weak group B variant phenotype B_m were present in Natal. Since the phenotypes of the four other Indians whose cells had reacted similarly and of the two Indians whose cells had reacted with anti-B in 'mixed field' patterns had not been shown by means of family studies to have been genetically inherited, their origin was uncertain and they were consequently called B_m-like and B_3-like respectively. The other possible causes of the characteristic

agglutination seen with the B_3-like cells, however, were discussed and shown to be unlikely in these two Indians. Moreover, direct evidence that the cells of one Indian had weak B antigen was obtained in tests with BS I lectin.

In my M.Sc. thesis (1976), weak group B variants had not been recorded in the Natal Negroes (Zulus) or in the Natal White controls, and it was therefore probable that the genes for the B_m (and possibly also for the B_3) phenotype had been brought to Natal from India by the Indian immigrants. The evident rarity of the weak group B variant phenotypes, both in Natal and in India, showed that they were unlikely to be due to local factors in the environment, and Lopez, Bouguerra, Lemeud, Badet and Salmon (1974) and Salmon (1976) are of the opinion, following the detailed quantitative studies of the former workers with weak group B variant cells and the same set of reagents, that the variants are genuinely-inherited genetic characters. Lopez *et al.* (1974) observed that the expression of the B antigen was invariably the same within a family but differed between families, and my findings in the Natal Indians described in this chapter support their view. Although my studies were made at different times, my standard anti-A, anti-B and anti-A,B reagents were obtained from a single commercial source which subjects them to rigorous quality control measures before issue, and my techniques have always been applied to every specimen investigated with the same stringent degree of personal care. It is of interest that the biochemists, who have studied the serum transferases in individuals with different weak group A variant, different weak group B variant and A, B and O H-deficient red cell phenotypes, also support the concept that they are genuinely inherited genetic characters (Watkins, 1980).

The Natal Indians described in this chapter spoke either Tamil or Telegu and not the Hindi Indian language expected to have been spoken by the non-Moslems with weak group B variant cells recorded from Maharashtra and Gujarat in India by Bhatia and Sathe (1974). This showed that the variants may occur in the Dravidian as well as in the Aryan language-speaking Indians.

5.6 SUMMARY

The weak group B variant phenotype B_m, or *Category 2* red cells, was described in 11 members of one and in two members of a second Natal Dravidian language-speaking Indian family. Within each family the B antigen expression of the cells was almost identical but between the families it was noted to vary minimally. Minor variations were also found in four further Natal Indians whose cells were described as *Category 2*- or B_m-like as family studies had not been made in their case. The reactions of the cells and

serum in two Natal Indians who had *Category 3-* or B_3-like cells, the B antigen in one being more weakly-expressed than in the other, were also described. The possibility that these Indians had group B and group O cell populations was discussed and in one Indian shown to be unlikely as his cells had weaker-than-normal B antigen.

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Study 4

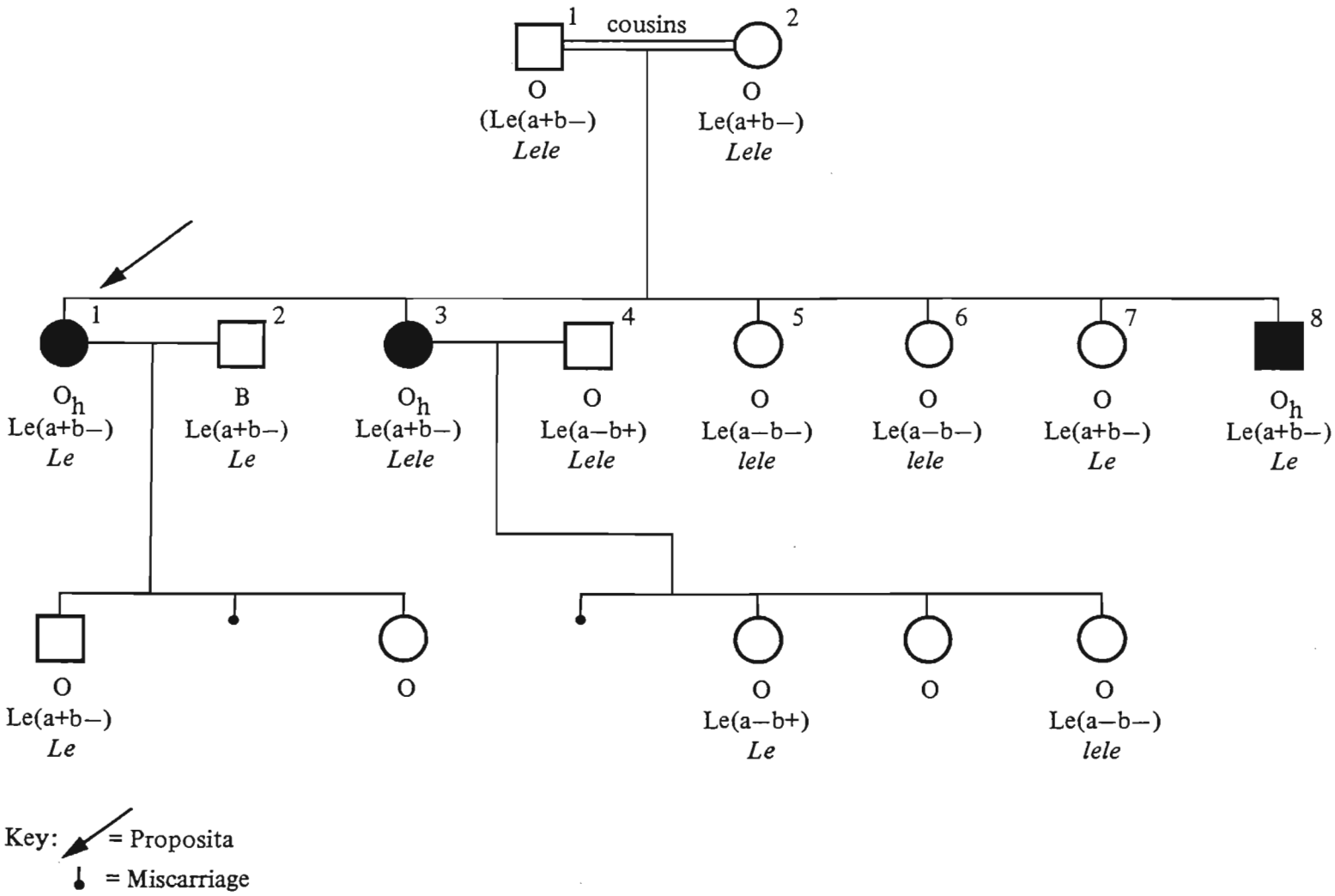
THE FIRST 'BOMBAY' O_h PERSON IN SOUTH AFRICA

7.1 INTRODUCTION

The O_h or 'Bombay' phenotype, discovered by Bhende, Deshpande, Bhatia, Sanger, Race, Morgan and Watkins in three Indians from Bombay in India in 1952, is characterised by no A, B or H antigens being present on the red cells and no A, B or H substances being secreted in the saliva. However, O_h people have normal but unexpressed *ABO* and secretor genes. This was demonstrated by Levine, Robinson, Celano, Briggs and Falkinberg in 1955 when they showed that the normally-expressed *B* and *Se* genes in the parents of an O_h American woman were not expressed in her but had been passed on by her to her children in whom they were being re-expressed normally. Aloysia, Gelb, Fudenberg, Hamper, Tippett and Race (1961) subsequently showed, almost certainly, that the A_1 gene in an American/French O_h woman, and certainly that the A_2 gene in two American/English O_h brothers, had also been transmitted unexpressed through them in this way. The *O* genes were described first as being unexpressed in two Natal O_h Indian sisters whose parents and children were all group O by Moores in 1972 (Moores, 1972b) (Figure 7.1). The absence of *A*, *B* and *H* gene expression in O_h people is now known to be due to the inheritance of rare *hh* instead of *HH* genes or a double dose of a rare recessive suppressor gene which inhibits *H* gene enzyme synthesis (Watkins, 1980).

The 'Bombay' O_h family reported by Levine *et al.* in 1955 showed that the expression of the *Le* gene was also affected in O_h people. In a figure in which the possible pathways of *ABO, Hh, Sese* and *Lele* gene interaction were displayed, Watkins (1965) explained that precursor substance was transformed by *Le*, but not by *lele* genes, into Le^a substance. The Le^a substance then underwent transformation by *H* and *Se*, but not by *H* and *sese* or by *hh* and either *Se* or *sese* genes, into H, Le^a and Le^b substances. An O_h person (who had *hh* genes) might therefore secrete Le^a substance or have untransformed precursor substance and, consequently, have either Le(a+b–) or Le(a–b–) cells respectively, but would not have Le(a–b+) cells. Further work has now shown that Le^b substance is a product of *H* and *Le* gene interaction and is not formed when *hh* genes have been inherited (Watkins, 1980).

Figure 7.1 Family pedigree of P. and B. Gov. in whom the O group is not expressed



The first 'Bombay' O_h person described in South Africa, Mrs P.N., was identified by Moores on 30th December, 1964. She was a Natal Indian woman, and her discovery was reported by Moores in 1965 in a paper presented at the South African Blood Transfusion Congress held from 30th April to 1st May in Durban. This chapter contains an account of her discovery and of the serological studies which confirmed that she was O_h.

7.2 CASE HISTORY

The Indian woman, Mrs P.N., who spoke Telegu, was born at Isipingo, a coastal town near Durban, in about the year 1910. The place of origin of her family in India was not known either by her or her relatives and, as far as she was aware, her parents had not contracted a consanguineous marriage. She was found, suffering from cervical carcinoma in a hospital in Durban, when her doctor requested compatible blood to correct her anaemia before radiation therapy was commenced. Although apparently group O, her serum contained a strong saline- and enzyme- reacting antibody and had agglutinated the cells of 124 units of group O blood in the cross-matching tests. The auto-antibody control and direct antiglobulin tests had both been negative.

Since difficulty was being experienced in finding compatible blood for Mrs P.N., her blood samples were referred to me for further study. Although aware that O_h red cells grouped as O with anti-A, anti-B and anti-A,B reagents and were distinguished from normal O cells by a negative reaction with anti-H, I had not had the opportunity to identify such cells myself before. I confirmed that Mrs P.N.'s red cells were group O and, having found that her serum agglutinated the cells of 14 adult O donors (selected for their different groups in eight blood group systems) and two O cord cell samples, tested her cells and those of a known group O person (myself) with a known example of anti-H from a group A_1 patient (we had no *Ulex* anti-H lectin at that time). The results showed that Mrs P.N.'s cells were not agglutinated while the control cells reacted well with this serum. The negative reaction of Mrs P.N.'s cells with anti-H was confirmed subsequently using two commercial and five further examples of human anti-H, but the supply of her cells was too small for distribution to other centres for confirmation of her O_h status. However, later, the cells of other Natal Indians which were not agglutinated by her serum, were confirmed as being O_h in many centres in Britain, the United States, France and other parts of the world (see Chapter 9).

7.3 MATERIALS AND METHODS

Blood samples were received from Mrs P.N. on two occasions, the first in 1964 and the second early in 1965. She was not given a blood transfusion during this period as no compatible blood had been found for her and the red cells of all the relatives in her extensive family tested, who now lived in Durban and the north coast towns of Stanger and Darnall, had been agglutinated by her serum. A sample of her saliva was received and processed as described in Chapter 2. The preparation of the cell suspensions, the methods of absorbtion, elution and titration used and the saliva inhibition technique will also be found in this chapter.

7.4 RESULTS

Figure 7.2 shows that Mrs P.N., II-2, had seven children. Among them, III-3, III-7 and III-8 were A_1, III-5 was B and III-2 and III-6 were A_1B. Unfortunately, as her parents and husband were no longer living, neither the identity of her unexpressed *ABO* genes, which might have been A_1O, *BO* or A_1B, nor of her unexpressed secretor genes, could be determined. Her groups were identified as follows:–

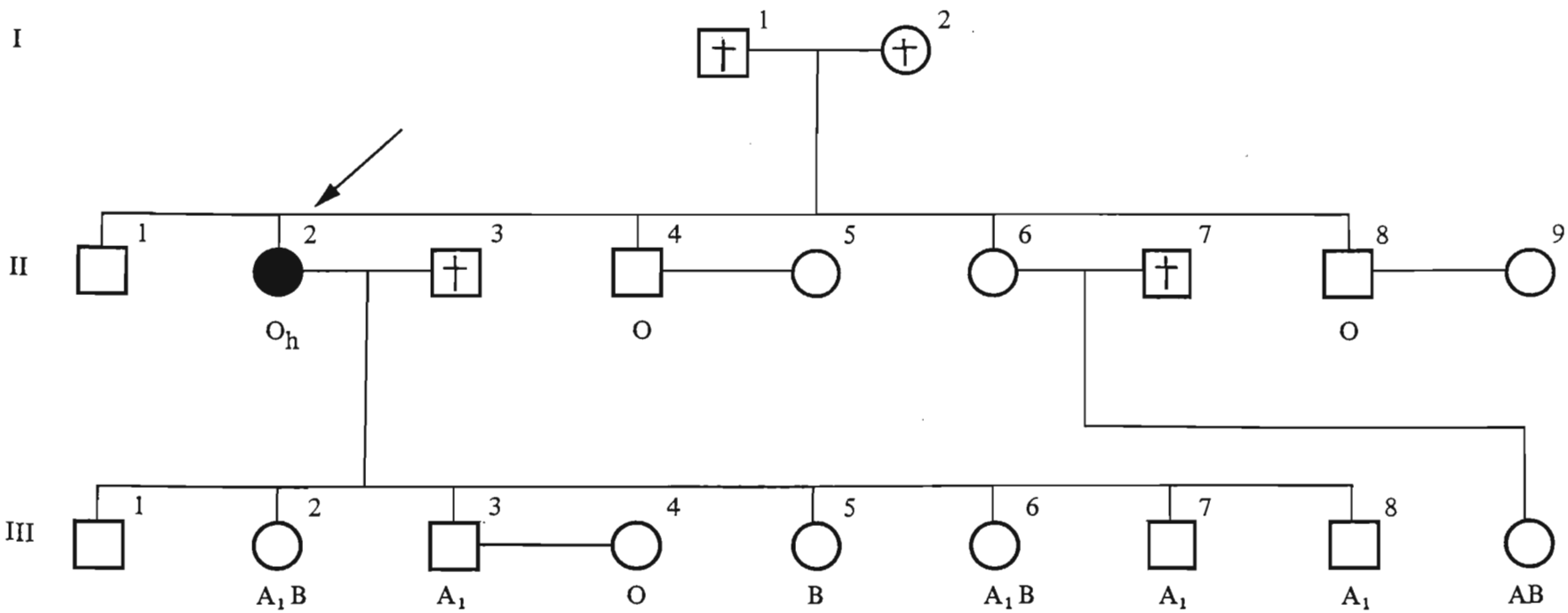
O_h, MNs, P_2, CDe/cde (Rh_1rh), K–, Le(a+b–), Fy(a+), 1+, Yt(a+)

Saliva inhibition titrations showed that Mrs P.N. did not secrete A, B or H substances (Table 7.1, part a).

In comparative saline and one-stage 0,5% bromelin titrations at 20°C, Mrs P.N.'s serum agglutinated A_1, B and O cells to titre 32, in one-stage 0,25% ficin titrations to titre 256 and in indirect antiglobulin titrations to titre 512 (Table 7.1, part b). The identity of the antibodies in her serum was confirmed as anti-A+B+H when, following the addition of equal volumes of the saliva of A, B and H secretors to all the appropriate test tubes in further titrations, the agglutination was seen to have been inhibited. Two absorbtions with A_1 cells left anti-B, two absorbtions with B cells left anti-A and two absorbtions with O cells left anti-A and anti-B in her serum (Table 7.1, part c). The titres obtained when the absorbed sera were subsequently titrated were not found to have been reduced by this procedure. The antibodies recovered by the 56°C heat technique of Landsteiner and Miller (1925) in the eluate from the A_1 cells, agglutinated B and O cells weakly, and those recovered in the

Figure 7.2

Part of the family pedigree of Mrs P.N.



Key: † = dead

↙ = proposita

TABLE 7.1

Results of tests with the saliva and serum of Mrs P.N.

Part a

Saliva inhibition titrations

	anti-A + A_2 cells						anti-B + B cells						anti-H + O cells					
	1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	8	16	32
Mrs P.N.	4	4	3	3	2	1	4	4	4	3	2	1	4	3	(3)	(±)	–	–
AH secretor	–	–	–	–	–	–	4	4	4	3	2	1	–	–	–	–	–	–
BH secretor	4	4	3	3	3	2	–	–	–	–	–	–	–	–	–	–	–	–
Non-secretor	4	4	3	2	2	1	4	4	4	3	2	1	3	2	(3)	(1)	–	–
Control saline	4	4	4	3	2	1	4	4	3	2	1	1	3	2	1	(3)	(1)	–

Part b

Serum	Technique at 20°C	Cells	Serum titrations dilutions 1	2	4	8	16	32	64	128	256	512	1024
Mrs P.N. O_h	Saline	A_1	4	4	3	2	(3)	(1)	–	–	–	–	–
		B	4	4	3	2	1	(2)	–	–	–	–	–
		O	4	4	4	4	2	1	(2)	–	–	–	–
	One-stage 0,5% bromelin	A_1	4	4	3	2	1	(3)	(1)	–	–	–	–
		B	4	4	3	2	1	(2)	(±)	–	–	–	–
		O	4	4	4	3	2	(3)	(1)	–	–	–	–
	one-stage 0,25% ficin	A_1	4	4	4	4	4	4	4	2	1	–	–
		B	4	4	4	4	4	4	4	2	±	–	–
		O	4	4	4	4	4	4	4	2	1	–	–
	indirect antiglobulin at 37°C	A_1	4	4	4	4	4	4	3	2	1	(2)	–
		B	4	4	4	4	4	4	2	1	(3)	(1)	–
		O	4	4	4	4	4	3	3	1	1	(2)	–

Part c

Serum of Mrs P.N. absorbed with:–		Serum titrations dilutions 1	2	4	8	16	32	64	128
A_1 cells	A_1	(2)	–	–	–	–	–	–	–
	A_2	(3)	(1)	–	–	–	–	–	–
	B	4	4	3	1	(2)	(±)	–	–
	O	(3)	–	–	–	–	–	–	–
B cells	A_1	3	2	1	1	(3)	(2)	–	–
	A_2	3	2	1	(3)	(2)	–	–	–
	B	(1)	–	–	–	–	–	–	–
	O	(2)	–	–	–	–	–	–	–
O cells	A_1	3	3	2	1	1	(2)	(1)	–
	A_2	2	2	1	1	(2)	(2)	(±)	–
	B	4	4	2	1	(3)	(2)	(±)	–
	O	(2)	–	–	–	–	–	–	–

TABLE 7.2

Natal Indians identified as O_h between the years 1964 and 1980

Year	Name	Category	*ABO* genes unexpressed	Lewis phenotype
1964	Mrs P.N.	Telegu	A_1O, B or A_1B	Le(a+b–)
	Miss S. Hari.			Le(a+b–)
1965	Miss G. Hari.	Hindi	BO or OO	Le(a+b–)
	Miss P. Hari.			Le(a+b–)
1967	Mrs P.C.	Telegu	A_1O, BO or OO	Le(a+b–)
	M. Gov.			Le(a+b–)
1967	D. Gov.	Tamil	OO	Le(a+b–)
	Miss A. Gov.			Le(a+b–)
1969	Mrs L. Dew.	Hindi	A_1O, BO or OO	Le(a+b–)
	G. Nai.		* A_1O	Le(a+b–)
1969	S. Nai.	Tamil	* BO	Le(a+b–)
	Mrs P. Gov.			Le(a+b–)
1970	Mrs B. Gov.	Tamil	OO	Le(a+b–)
	T. Gov.			Le(a+b–)
	Miss N. Moon.			Le(a+b–)
1970	B. Moon.	Tamil	OO	Le(a–b–)
	V. Moon.			Le(a–b–)
	Miss R. Gan.			Le(a+b–)
1972	Mrs K. Ram.	Hindi	?	Le(a+b–)
	Mrs D. Oma.			Le(a+b–)
1974	Mrs D. Rug.	Hindi	?	Le(a+b–)
	A. Nar.		* BO	Le(a+b–)
1976	S. Red.	Telegu	* BO	Le(a+b–)
	R. Red.		* A_1O	Le(a+b–)

* Determined by Dr W.M. Watkins from transferases in their sera.

eluates from the B and O cells agglutinated O cells weakly. These antibodies were later identified as anti-B+H and anti-H respectively.

7.5 DISCUSSION

The realization that Mrs P.N. was an O_h Indian established that the *h* gene was present in South Africa. In due course, other O_h persons were expected to be found there, and the importance of being able to locate compatible blood donors for them in an emergency led me to make a full family study whenever one appeared. As a result, 24 O_h Indians have now been identified in Natal (Table 7.2). An Indian in the Transvaal and two persons of mixed race in the Cape Province have also been confirmed by me as O_h from blood samples sent by colleagues in other blood transfusion services. However, not all the O_h Indians in Natal were suitable as blood donors and some of them are still young children.

As anticipated in 1964, Mrs P.N. had Le(a+b–) red cells. However, three Natal O_h Indians and one of the two O_h Coloured people identified in the Cape Province have since been found to have Le(a–b–) cells. By giving approximately equal titres with A_1, B and O cells in titrations made by four serological techniques, the anti-A+B+H antibodies in Mrs P.N.'s serum showed that she belonged to the category of 'typical' O_h individuals. The absorbtions with A_1, B and O cells confirmed that her serum contained separable anti-A, anti-B and anti-H, but the eluates recovered subsequently from these cells revealed that her serum contained more than a simple mixture of these antibodies. This subject is returned to and discussed in more detail in Chapter 11.

7.6 SUMMARY

The red cells of the first Natal O_h Indian described in South Africa were not agglutinated by anti-A, anti-B or anti-H and she secreted no A, B or H substances in her saliva. Her serum contained anti-A+B+H antibodies which agglutinated A_1, B and O cells to almost identical titres. Absorbtion-elution studies suggested that her serum contained more than a simple mixture of anti-A, anti-B and anti-H antibodies.

Table 7.2 shows the Indians identified as O_h in Natal between the years 1964 and 1980.

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Paper 26a

Further examples of O_h Le(a–b–) people have been found: the propositus in an Italian family (Gandini *et al.*[550], 1968); two sibs in a Canadian family (Pretty *et al.*[543], 1969); three sibs in a Natal Indian family (Moores[544], 1972). The last two families were particularly useful: that from Canada[543] strongly suggested that the *Hh* genes were not controlled from the *Le* locus and that from Natal[544] proved it. In the Natal family the father is *Hh lele* and the mother *Hh Lele*; three of the children are O_h, one of them *hh Lele* and two *hh lele*, at least one child must reflect recombination between the two loci.

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Blood Groups in Man.
by R.R. Race and R. Sanger; 6th edition, Blackwell Scientific Publications, Oxford, 1975, p23 and 85.

Paper 26b

THE "BOMBAY" BLOOD-TYPE IN NATAL. P.P. Moores, The Natal Blood Transfusion Service, Durban, South Africa.

Eighteen examples of the "Bombay" blood-type have been found in nine unrelated Natal Indian families in a total population of about 500,000. One family of two group O parents had three O Le(a-b-), two "Bombay" Le(a-b-), one O Le(a+b-) and two "Bombay" Le(a+b-) children.

The "Bombay" anti-H of one male and three female adults was an IgG-type antibody, probably immune in origin.

Haemolytic disease of the newborn was clinically and serologically demonstrable in the infant of one "Bombay" mother, and in another was demonstrated only serologically.

Abstract: 25th Annual Meeting of the American Association of Blood Banks and 13th Congress of the International Society of Blood Transfusion, Washington, 1972, p11.

Study 5

RED CELL AND SERUM STUDIES IN THE FIRST FAMILY TO HAVE BEEN RECORDED IN WHICH BOTH O_h Le(a+b–) AND O_h Le(a–b–) SIBLINGS WERE REPRESENTED

8.1 INTRODUCTION

Watkins (1959), Watkins and Morgan (1959) and Ceppellini (1959) described a theory of biosynthesis of the A, B, H and Lewis substances which made provision for a then unknown O_h Le(a–b–) red cell phenotype. The theory supposed that a precursor substance was transformed by the sequential activity of *Lele, Hh, Sese* and *ABO* genes into blood group antigens on the red cells and substances in the secretions. When an *Le* and *hh* gene had been inherited, the red cell phenotype was O_h Le(a+b–) and the individual secreted Le^a substance only, and when *lele* and *hh* genes had been inherited, the phenotype was O_h (Le(a–b–) and neither ABO nor Lewis substances was secreted. The investigations made by these and other workers which followed showed that the genes were more likely to act in the order *Sese, Hh, ABO* and *Lele* (Boettcher, 1978), but the possibility apparently also exists that independent recessive suppressor genes inherited in double dose may alter the α-2-L-fucosyltransferase expressed by normally-inhertied *H* genes in O_h individuals (Watkins, 1980).

The first known O_h Le(a–b–) individual was a Bengali Moslem from East Pakistan (Giles, Mourant and Atabuddin, 1963). As predicted, his red cells, which were not agglutinated by anti-A, anti-B, anti-A,B or by anti-H, were also not agglutinated by several examples of anti-Le^a, anti-Le^b, anti-Le^a+Le^b and one example of anti-Le^x reagents. Moreover, these reagents were also not inhibited on being mixed with his saliva. Unfortunately, the family study provided no information as to whether or not the *Hh* and *Lele* genes had been inherited independently in his family members.

Further examples of the O_h Le(a–b–) phenotype were recorded by Bond, Shirgaonkar, Randeria and Bhatia (1965), by Gandini, Sacchi, Reali, Veratti and Menini (1968) and by Pretty, Taliano, Fiset, Baribeau and Guévin (1969), but the Lewis types in the family described by Gandini *et al.* (1968) were questioned by Wiener (1969) who calculated that five Le(a–b–) among six children born to *Lele* parents

was likely to occur only once in 222 times. The family described by Pretty *et al.* (1969) strongly suggested, but failed to prove, that the *Hh* genes were not controlled from the *Le* locus (Race and Sanger, p 23, 1975), but the groups in the first family containing both O_h Le(a+b–) and O_h Le(a–b–) siblings, that of the Natal Indians, Mr and Mrs Moon. (Moores, 1972b) described here, confirmed that *h* segregated independently of the *Le* and *le* genes (Race and Sanger, p 23, 1975). In 1973 and 1976, additional Indian families containing both O_h Le(a+b–) and O_h Le(a–b–) members were reported by Sathe and Bhatia, and their 1976 family contained O_h Le(a–b–) members in two generations. The groups in these families provided further confirmation that the *Hh* and *Lele* genes segregated independently.

As the rare gene *h* (originally the *x* gene of Levine, Robinson, Celano, Briggs and Falkinberg, 1955), when homozygous, prevents expression of the *ABO* secretor genes, it was of interest in this study to determine whether or not the apparently heterozygous *Hh* parents of O_h persons had reduced H antigen expression on their red cells. The cells of members of the Moon. family were convenient for this purpose as the parents were both group O and different amounts of H, due to *ABO* genes of other types inherited, could be ignored. The apparently small amount of H antigen on enzyme-treated O_h cells, that had been detected with *Ulex europaeus* and *Cytisus sessifolius* anti-H lectins by Dodd and Lincoln (1978), was also investigated using *Ulex* anti-H, the anti-A+B+H antibodies of a number of Natal O_h Indians and the cells of the O_h members in the Moon. family and of controls.

8.2 CASE HISTORY

The O_h phenotype of the propositus in the Natal Indian family, Moon., V. Moon., was discovered in 1969 while he was in hospital suffering from an undisclosed illness. He was then three years old and no compatible blood for a blood transfusion had been found for him. A unit of blood was donated by a known Natal O_h Indian donor, and V. Moon.'s subsequent recovery was uneventful.

The blood groups in the Moon. family were studied in 1970 and again in 1973. On each occasion, blood samples were obtained from all the family members, and in 1973 saliva samples were obtained from them as well. In 1975, four family members each donated 100 mls of their blood for the preparation of ethanol soluble substances (ESS). The studies revealed that both V. Moon. and a sibling had phenotype O_h Le(a–b–) cells and that another sibling had phenotype O_h Le(a+b–) cells.

8.3 MATERIALS AND METHODS

The red cells and saliva for this study were prepared for use as indicated in Chapter 2. The reagents used consisted of several examples of standardised anti-A,anti-B and anti-A,B, *Ulex* anti-H lectin, anti-Le^a, anti-Le^{bL} (not inhibited by the saliva of a secretor of H but no Lewis substances), anti-Le^x, and antibodies of other specificities for determining the groups of the cells. The anti-A+B+H sera used were from several known Natal O_h Indians.

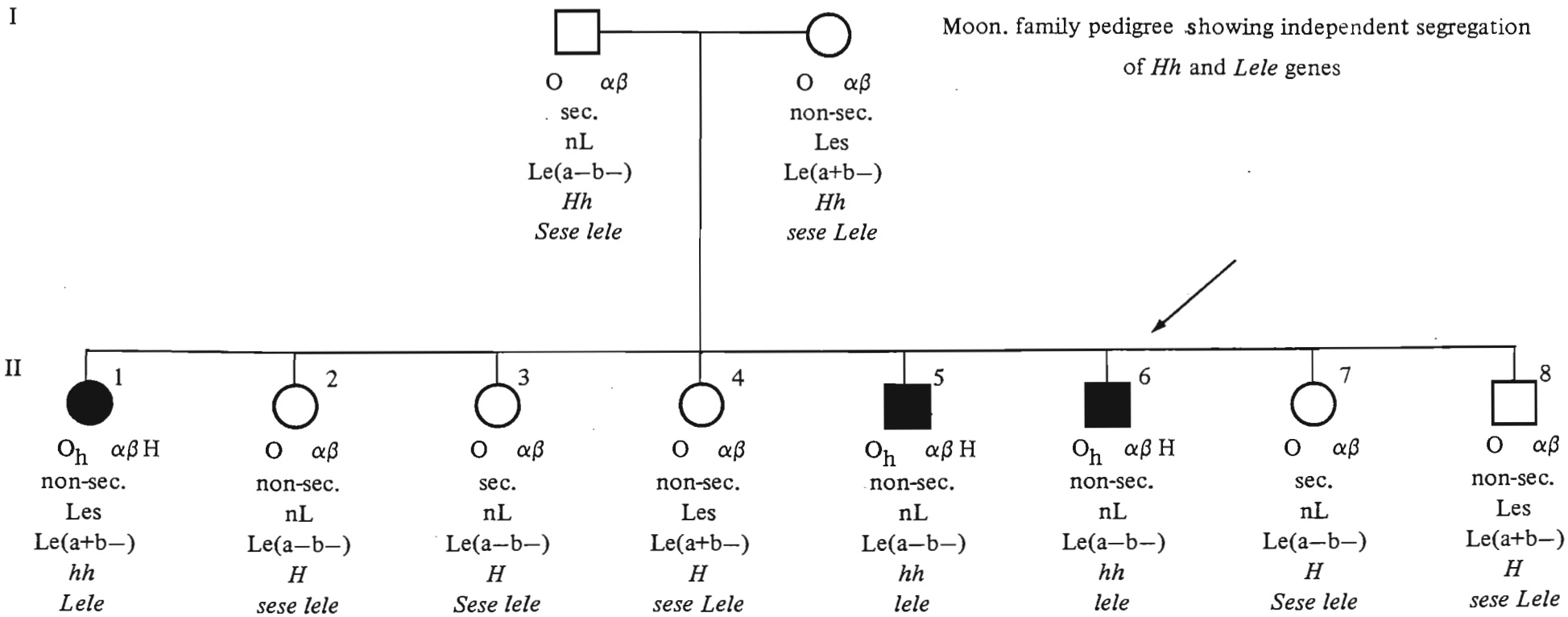
8.4 RESULTS

8.4.1 The family red cell and saliva groups

The red cells of the parents, I-1 and I-2, in the Moon. family (Figure 8.1), both of whom were group O, were agglutinated by *Ulex* anti-H lectin and by 'Bombay' anti-A+B+H sera, and their sera contained normal anti-A,B allo-antibodies. The cells of I-1 typed as Le(a−b−) and those of I-2 as Le(a+b−), and the saliva of I-1 inhibited *Ulex* anti-H only while the saliva of I-2 inhibited anti-Le^a only. I-1 was therefore a secretor of H substance and possessed *Se* and *lele* genes, while I-2 was a secretor of Le^a substance and possessed *sese* and *Le* genes. Among their eight children, the cells of II-1, II-5 and II-6 were not agglutinated by anti-A, anti-B, anti-A,B, anti-A+B+H or *Ulex* anti-H lectin, and their sera contained anti-A+B+H. The phenotype of their cells was therefore O_h. The cells of II-1 typed as Le(a+b−) and the cells of II-5 and II-6 as Le(a−b−). The saliva of II-1 inhibited anti-Le^a, while the saliva of II-5 and II-6 failed to inhibit anti-A, anti-B, anti-H, anti-Le^a or anti-Le^{bL} reagents. II-1 had therefore inherited an *le* gene from her father, I-1, and an *Le* gene from her mother, I-2, while II-5 and II-6 had inherited *le* genes from both I-1 and I-2. As II-2, II-3 and II-7 were non-secretors of Lewis substances and their cells were (Le(a−b−), I-2 was seen to be heterozygous *Lele*, and by not secreting H substance in their saliva, II-2, II-4 and II-8 confirmed that I-1 had heterozygous *Sese* genes. Provided that the O_h phenotype was due to the inheritance of *hh* genes (Watkins and Morgan, 1955), the inheritance by II-1 of *h* and *le* from her father, I-1, and of *h* and *Le* from her mother, I-2, and the inheritance by II-5 and II-6 of *h* and *le* from both I-1 and I-2, showed that the single *h* gene in I-2 had travelled independently of the *Lele* genes to these three children (Race, 1973).

Figure 8.1

Moon. family pedigree showing independent segregation of *Hh* and *Lele* genes



Key: ↙ = Propositus, V. Moon.
nL = No Lewis substances secreted
Les = Le^a substance secreted

8.4.2 The H antigen dosage studies

8.4.2.1 In titrations with various reagents

Since 'Bombay' O_h Indians are homozygous for the *h* gene (Watkins and Morgan, 1955) or homozygous for recessive suppressor genes inherited independently of the *ABO* genes (Watkins, 1980), it was not unreasonable to suppose that their parents and some of their siblings might be heterozygous for these genes. To determine whether the cells of heterozygous (*Hh*) individuals carried less H antigen than those of homozygous (*HH*) individuals, carefully matched cell suspensions from all the Moon. family members and a known control (PPM) were tested in strict parallel titrations with *Ulex* anti-H lectin and the anti-A+B+H antibodies of ten Natal O_h Indians. Five of the anti-A+B+H antibody donors were females, two of whom, as their infants had become jaundiced in the neonatal period, may have been stimulated antigenically, two were non-transfused males and the remaining three were the O_h children in the Moon. family. The anti-A+B+H antibodies of V. Moon., the three-years old propositus in this family, had the highest titres so far recorded in the laboratory. As far as it could be ascertained by testing the sera of the O_h donors with the cells of each other, their sera contained no other blood group antibodies.

Table 8.1 lists the results obtained with the *Ulex* anti-H lectin and two of the ten ant-A+B+H sera. The results with the remaining eight anti-A+B+H sera are not shown in this table as they were essentially the same. The *Ulex* and the anti-A+B+H sera detected no differences in H antigen strength between the cell samples tested or between them and the control 0 cells. The weaker H antigen expected on heterozygous *Hh* cells was therefore either not distinguished by the reagents used or both heterozygous *Hh* and homozygous *HH* cells carried the same amount of H antigen.

8.4.2.2 Using *H* gene-specified enzymes

In the second study, Dr W.M. Watkins kindly assayed the α-2-L-fucosyltransferase activities in the sera of the parents, one O and one O_h sibling of the Moon. family, and in four O controls who consisted of a White woman and three Natal Indian males. The assay was made by measuring the amount of radioactive [^{14}C] fucose incorporated into a low molecular weight acceptor. Although the results, shown in Table 8.2, suggested that a dosage effect existed between the parents (I-1 and I-2, Figure 8.1) and the controls, the intermediate level in the serum of II-2, who could

TABLE 8.1

H-antigen dosage titrations with red cells of Moon. family members

Cells	ABO Group	Suggested genes	cells in saline — Ulex anti-H dilutions									cells ficin-treated — Ulex anti-H dilutions									cells in saline — anti-A+B+H of Mrs P.N. 30 dilutions									cells ficin-treated — anti-A+B+H of Mrs P.N. 30 dilutions									cells in saline — anti-A+B+H of V. Moon. dilutions										cells ficin-treated — anti-A+B+H of V. Moon. dilutions									
			2	4	8	16	32	64	128	256	512	2	4	8	16	32	64	128	256	512	8	16	32	64	128	256	128	256	512	1024	2008	8	16	32	64	128	256	128	256	512	1024	2008	4096
I-1	O	Hh	4	1	±	–	4	3	2	1	–	4	3	4	3	1	(2)	–	4	3	1	–	4	3	3	1	±	–	4	3	2	1	±	–	4	4	3	2	1	±	–						
I-2	O	Hh	4	2	±	–	4	3	2	1	–	4	2	4	2	1	(2)	–	4	2	1	–	4	3	3	1	±	–	4	3	2	1	±	–	4	4	3	2	1	±	–						
II-1	O_h	hh	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
II-2	O	H	4	1	±	–	4	3	2	1	–	4	2	4	2	1	(2)	–	4	2	1	–	4	4	4	1	±	–	4	3	2	1	±	–	4	4	4	2	1	±	–						
II-3	O	H	4	1	±	–	4	3	2	1	–	4	2	4	2	1	(2)	–	4	2	1	–	4	4	3	1	±	–	4	3	2	±	–	–	4	4	3	2	1	±	–						
II-4	O	H	4	1	±	–	4	3	2	1	–	4	3	4	3	1	(2)	–	4	2	1	–	4	4	2	1	±	–	4	3	2	1	±	–	4	4	3	2	1	±	–						
II-5	O_h	hh	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
II-6	O_h	hh	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
II-7	O	H	4	1	±	–	4	3	2	1	–	4	2	4	2	1	(2)	–	4	2	1	–	4	4	2	1	±	–	4	3	2	1	±	–	4	4	2	1	±	–	4	3	2	1	±	–	–
II-8	O	H	4	1	±	–	4	3	2	1	–	4	2	4	2	1	(1)	–	3	1	±	–	4	4	2	1	±	–	4	3	1	±	–	–	4	4	2	1	±	–	4	3	1	±	–	–	–
Control	O	HH	4	1	±	–	4	3	2	1	–	4	2	4	2	1	(2)	–	4	2	1	–	4	4	2	1	±	–	4	2	2	1	–	–	4	4	2	1	±	–	4	2	2	1	–	–	–

TABLE 8.2

Assay of 2′-fucosyltransferase activity in the serum of Moon. family members

Details supplied by W.M. Watkins

Subject	Group	Genotype	2′-Fucosyltransferase activity (% incorp. [^{14}C] fucose
I-1	O	*Hh*	6,7
I-2	O	*Hh*	6,6
II-1	O_h	*hh*	nil
II-2	O	*HH* or *Hh*	8,1
Control S.N.	O	*HH*	10,5
Control A.K.	O	*HH*	10,7
Control S.B.	O	*HH*	10,2
Control P.P.M.	O	*HH*	10,6

have been either homozygous *HH* or heterozygous *Hh*, unfortunately rendered the findings inconclusive.

8.4.2.3 Using red cell substances

Vos, Moores, Downing and Mohideen (1976) described the isolation of ethanol-soluble substances (ESS) from human and animal red cells and reported that the ESS obtained from the cells of Gov. (G. Nai.), a Natal 'Bombay' O_h Indian, inhibited the agglutination of O cells by 'Bombay' anti-A+B+H antibodies weakly. The inhibition detected suggested that O_h cells had 'hidden' H antigen. In a later study (Vos and Moores, 1976), the ESS obtained from the cells of seven further Natal O_h Indians and controls were found to vary in activity from person to person. I-1, I-2, II-1 and II-2 in the Moon. family were included in these tests, but the ESS obtained from I-1, an obligatory *Hh, lele*, secretor of H substance, inhibited the anti-A+B+H antibodies of six other Natal 'Bombay' Indians to the same titre as the *HH, lele* and *HH, Le* controls who secreted H substance. Similarly, the ESS of I-2, an obligatory *Hh, Le* non-secretor of H substance, inhibited the anti-A+B+H antibodies to the same titre as the *HH, Le* non-secretor control. The findings suggested, either that the cells of the *Hh* members in this family had as much H antigen as the controls, or that the method used was not suitable for H antigen dosage studies. The ESS of II-2, who was an *HH* or *Hh, lele*, non-secretor of H substance, inhibited the anti-A+B+H antibodies to approximately the same titre as the ESS of her mother (I-2); and the ESS of II-1, who had *hh, Le* genes, inhibited these antibodies only minimally.

The following tests were made by myself as part of this study. The anti-A+B+H antibodies in a sample of serum from M. Gov. (167), and the anti-H lectin of *Ulex europaeus*, were standardised for inhibition tests by titrating them with ficin-treated O cells. The dilution selected for use was the highest which showed 4+ agglutination of these cells. Equal volumes of the reagents were mixed with the ESS of I-1, I-2, Mrs B. Gov. (a Natal O_h Indian control) and an O control (PPM), and inhibition was allowed to take place for 30 minutes at 20°C. One volume of ficin-treated O cells was then added, and the tests were read after a further hour at 20°C. In the second study, which was conducted in parallel, the *Ulex* anti-H lectin was titrated, and equal volumes of all the dilutions were tested for inhibition. In the third study, the ESS were titrated, and a volume of each dilution was mixed with the *Ulex* anti-H lectin which had been diluted 1 in 5 with saline. The inhibition test was then completed.

Table 8.3 shows the findings in this study. The ESS of I-1, I-2 and the control inhibited both the anti-A+B+H antibodies and the *Ulex* anti-H lectin, and the ESS of Mrs B. Gov. inhibited both partially (Table 8.3, part a). When the *Ulex* was titrated, the ESS of I-1 and I-2, both of whom were thought to have *Hh* genes, inhibited this reagent to the same degree as the ESS of the *HH* control; and when the ESS were tirated, the titres of these substances in the three individuals were all seen to be similar. By contrast, the ESS of Mrs B. Gov. inhibited the *Ulex* anti-H to a lesser extent, and its titre was also lower than those of the other three ESS (Table 8.3 part b). Although confirming that *HH* and *hh* cells were distinguishable from each other in these tests, the results therefore failed to show that the presumed *Hh* cells of I-1 and I-2 had less H antigen than the *HH* control cells.

8.4.3 Investigation of the H antigen apparently detected on the cells of a Natal O_h Indian

In 1974, using the cells of a Turkish O_h girl, Poschmann, Fischer, Seidl and Spielman suggested that H existed in the form of a cryptantigen on O_h red cells. Two years later, Vos and Moores (1976) suggested that molecules having H specificity were hidden within the red cell membranes of certain Natal O_h Indians, and in 1978, Dodd and Lincoln obtained very high haemagglutination titres with some preparations of *Ulex* anti-H lectin after the cells of 'W'. (A. Nar.), a Natal O_h Indian, and of other O_h persons had been treated with enzymes. Dodd and Lincoln (1978) suggested that the successful detection of H antigen on O_h cells required the use of 'incomplete' anti-H lectins, and these serologists also recorded that the anti-A+B+H antibodies of 'W'. and of another O_h individual had cross-reacted weakly with each others' cells in saline tests. In addition, slight auto-agglutination of their enzyme-treated cells had been recorded.

Prompted by these findings, a study was made of the reactions of bromelin-, ficin- and Löw 's-enzyme-treated O_h cells with anti-A+B+H sera from 19 Natal O_h Indians, including the sera of 'W'. (A. Nar. 202) and the three O_h members in the Moon. family. Four examples of *Ulex* anti-H lectin, examples of commercial and eel anti-H, human anti-H (group B) and anti-HI, and an extract of snail *Terebralia* were included as well. The object of the study was to discover whether reactions similar to those recorded by Dodd and Lincoln (1978) could be detected, and whether or not such agglutination could be inhibited with the saliva of a secretor of H but of no Lewis substances. The tests were made with bromelin- and ficin-treated cells, and also with

TABLE 8.3

Results of inhibition tests using ethanol-soluble substances (ESS) extracted from Hh, hh and HH cells of various Moon. family members and of control cells by Dr G.H. Vos

Part a

Subject	Group	Presumed genotype	Substance	Reagents: Ulex anti-H (O cells)	Reagents: anti-A+B+H (O cells)
I-1	O	*Hh*		–	–
I-2	O	*Hh*	ESS	–	–
Mrs B. Gov.	O_h	*hh*		2	1
Control (PPM)	O	*HH*		–	–
Saline				4	4

Part b

Subject	Group	Presumed genotype	Substance	Ulex anti-H dilutions								Ethanol-soluble substance dilutions						
				1	2	4	8	16	32	64	128	1	2	4	8	16	32	64
I-1	O	*Hh*		(3)	–	–	–	–	–	–	–	–	–	(3)	1	3	4	4
I-2	O	*Hh*	ESS	(3)	–	–	–	–	–	–	–	–	–	(±)	1	3	4	4
Mrs B. Gov.	O_h	*hh*		1	(3)	–	–	–	–	–	–	(3)	1	1	4	4	4	4
Control (PPM)	O	*HH*		(2)	–	–	–	–	–	–	–	–	–	–	(2)	2	4	4
Saline				4	4	4	3	(3)	(1)	–	–	4	4	4	4	4	4	4

Löw 's enzyme utilized in a one-stage test, as described in Chapter 2.

Table 8.4 is a summary of the findings obtained in these tests. The anti-A+B+H antibodies of Mrs L. Dew. (219), Mrs K. Ram. (254), Mrs R. Gan. and V. Moon. agglutinated the bromelin- and ficin-treated O_h cells weakly but, where the other results are shown as positive, the cells appeared merely to be aggregated. The H secretor saliva could not be said with certainty to have inhibited the reactions of any of the anti-A+B+H antibodies with the bromelin- or ficin-treated O_h cells, and no agglutination or aggregation of the O_h cells was detected in the presence of Löw 's enzyme. High titres were not obtained either with the bromelin- or the ficin-treated O_h cells, with these cells and Löw 's enzyme or with the four examples of *Ulex* anti-H lectin, but the anti-A+B+H antibodies which had reacted most strongly with the ficin-treated O_h cells were noticed to be those that had the highest titres against the untreated O cells.

8.5 DISCUSSION

In the first part of this chapter, it was shown that the Moon. family, which contained both O_h Le(a+b–) and O_h Le(a–b–) members, had made an important contribution towards our knowledge of the way in which the *Hh* and *Lele* genes are inherited by having kindly provided samples of their blood and saliva. In the second part, the H antigen dosage titrations with the cells of the presumed obligatory *Hh* parents in this family showed that their cells were indistinguishible from the cells of their five group O children, at least one of whom might have inherited *HH* genes, and from the known *HH* control cells. This finding was supported by the lack of significantly different amounts of α-2-L-fucosyltransferase activity detected in the family members' and control sera tested, and by the similar quantities of ethanol-soluble substances (ESS) extracted from the family members' and the control cells. While it was known that not all antibodies were capable of showing dosage, even in the MNSs system where many showed it regularly, these findings, which suggested that the parents might have *HH* genes, surprisingly lent some support to the view (see Watkins, 1980, p 58) that the absence of H antigen on O_h cells might be due to the inheritance of two recessive suppressor genes at a separate locus, rather than to the inheritance of *hh* genes.

In the third part of this chapter, the findings showed that anti-H specificity had not been confirmed as being the reason for the weak agglutination of two-stage enzyme-

TABLE 8.4

Tests with anti-A+B+H and other reagents using enzyme-treated cells from Natal O_h Indians

Reagent	antibody	Titre with untreated		Titre with ficin-treated		Titre with brom.-treated		Titre with O_h cells, Löw 's enzyme and	
		O cells	O_h cells	O_h cells and saliva	O_h cells and saline	O_h cells and saliva	O_h cells and saline	saliva	saline
Mrs P.N. 30	anti-A+B+H	64	0	±	1	1	1	0	0
Mrs P.C. 121	,,	16	0	0	±	0	0	0	0
G. Nai. 135	,,	32	0	0	0				
S. Nai. 212	,,	32	0	0	0				
Mrs D. Rug. 197	,,		0	1	2	0	1	0	0
Mrs D. Oma.	,,	128	0	0	1				
A. Nar. 202	,,		0	0	1				
S. Red. 215	,,		0	0	±				
R. Red. 216	,,		0	1	1				
M. Gov. 213	,,	16	0	0	0	0	0	0	0
Miss A. Gov. 214	,,		0	0	1				
Mrs L. Dew 219	,,	128	0	±	±	1	1	0	0
Mrs P. Gov. 230	,,	32	0	1	1				
Mrs B. Gov. 231	,,	64	0	±	±				
Mrs K. Ram. 254	,,	256	0	0	4	0	1	0	0
Mrs R. Gan.	,,	512	0	4	8				
N. Moon. II-1	,,	128	0	±	1				
B. Moon. II-5	,,	64	0	±	1				
V. Moon. II-6	,,	256	0	1	2	1	1	0	0
S. Mhlo.	anti-H	0	0	0	1				
M. Molet.	anti-HI	64	2	8	8				
S. Khan.	anti-HI	16	0	1	8				
Ulex 189	anti-H	32	0	0	2	0	0	0	4
Ulex 300	,,	32	0	0	0	0	0	0	0
Ulex 381	,,	32	0	0	0	0	0	0	0
Ulex R343	,,	32	0	0	0	0	0	0	0
Commercial	,,		0	1	8				
Eel	,,		2	4	>8				
Terebralia	unknown	>16	2	>8	>8				
Peanut	anti-T	0	0	0	0				
Control	D^u serum	0	0	>8	>8	>8	>8	>8	>8
Control	AB serum	0	0	0	0	0	0	0	0

treated O_h cells seen in the tests with anti-A+B+H antibodies. However, as the anti-A+B+H sera which had the highest titres had given the strongest reactions, the activity seemed to be a property of the sera, rather than of the cells. Perhaps the sera of O_h people contained a hitherto unsuspected antibody which had the capacity to react with an antigen exposed on the membranes of enzyme-treated O_h cells. Alternatively, enzyme-treated O_h might be more liable than O cells to aggregate non-specifically in the presence of some sera and lectins, especially the sera which contained highly concentrated antibody molecules, and the method of enzyme-treating these cells might therefore require some adjustment.

8.6 SUMMARY

Studies with the red cells and sera of members of the Moon. family showed that the gene *h* was inherited independently of the *Le* and *le* genes. The normal anti-H titres and normal amounts of ethanol-soluble substances (ESS) obtained from the cells, and the normal amount of H transferase activities detected in the sera, of the apparently *Hh* parents in this family showed that their cells and sera could not be distinguished by these methods from those of their group O children and the *HH* controls. The weak agglutination seen with the enzyme-treated cells of the O_h members in this family and in the families of other Natal O_h Indians using their own anti-A+B+H antibodies and those of others, which was not inhibited by H substance, was not thought to be due to serologically detectable H antigen on their cells.

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Hemagglutination Inhibition Studies for the Evaluation of Blood Group Antigens in Ethanol Soluble Substances (ESS) Obtained from Human, Baboon and Vervet Monkey Red Blood Cells

G. H. Vos, P. P. Moores, H. J. Downing, and A. F. C. Mohideen

From the Natal Institute of Immunology, Durban, South Africa

Soluble blood group substances, isolated from the red blood cells of humans, baboons, and vervet monkeys by ethanol extraction, possessed serologically active specificities for the following antigens: A, B, H, Le^a, Le^{bL}, P, P_1, P^k, and I. Human red blood cells lacking any of these specificities by the direct hemagglutination test also lacked the related antigens in their soluble extract. The only exception was in 'Bombay' Oh cells, from which soluble H substance could be readily isolated. Soluble substances obtained from baboon and vervet monkey red blood cells, which lack the human variety of A, B, and H antigens on their red blood cells, inhibited both human and lectin anti-H reagents. The detection of 'hidden' H activity in Oh cells will pose some important questions regarding membrane characteristics and the role of immune surveillance.

The investigations of Morgan,[12] Watkins,[20] and Kabat[8] greatly advanced our knowledge of the chemistry of blood group substances in glandular tissues and their secretions. Unfortunately, information regarding cellular blood group substances has remained scanty. In this study, a procedure for obtaining ethanol soluble substances (ESS) from the red blood cells is described for the evaluation of blood group determinants on cells. The aim of the investigation was to examine the distribution and characteristics of a variety of well-defined human red blood cell antigens and to assess whether similar determinants could also be extracted from the red blood cells of baboons and vervet monkeys.

Since our findings are likely to depend on the constitution of the glycolipids involved, it would be of great interest if they could be followed up on the lines of the outstanding work of Watkins and Morgan[19] on the biosynthesis and immunochemistry of blood group specific determinants. Such a study lies outside the scope of the present investigation.

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Materials and Methods

Blood samples were obtained in ACD solution (acid-citrate-dextrose) from healthy adult donors with various blood groups including one 'Bombay' Oh and two Tja negative ($P + P_1 + P^k$ negative) donors. For the study of nonhuman primate red blood cells it was necessary to use pooled samples of blood in order that a minimum of 100 ml of washed packed cells could be obtained for ethanol fractionation. The cells were washed before pooling and only bloods from AH or BH secretor baboons and vervet monkeys were combined so that each 100 ml unit of packed cells represented a mixture of cells from the same secretor type. The chacma baboons were of the species *Papio ursinus*, Kerr and the vervet monkeys of the species *Cercopithecus pygerythrus*, Cuvier.

Preparation of Red Blood Cell Suspensions

Fidalgo, Katayama, and Najjar[5] found that two types of gammaglobulin bind to autologous human red blood cells and that this protein coat can be removed from the membrane by washing with 0.15M NaCl. We used this procedure to ensure that only antigenic determinants related to the red blood cell membrane were recovered and not a combination of membrane and serum protein factors. We speculated that the removal of surface bound protein from the red blood cells might demonstrate the relationship of the Lewis antigens which are said to be acquired from the plasma and should therefore not be intimately associated with the cell membrane.[7,10,18]

Ethanol Soluble Red Blood Cell Substances (ESS)

To a 100 ml suspension of washed packed cells was added 400 ml of 95 per cent purified ethanol. The mixture was placed in a 500 ml container designed for a Vertis blender. It was homogenized for one minute at 60,000 rpm four times/hour between the hours 9 a.m. and 5 p.m. After standing at room temperature overnight, the red blood cell debris was removed by centrifugation and the supernatant ethanol was placed in a 2 liter glass beaker. Sodium chloride was added and the undissolved salt removed. The beaker containing the ethanol was then placed on a mechanical stirrer inside a 4 C refrigerator. Four volumes (1.6 liters) of cooled acetone were slowly added to the ethanol and the white precipitate which formed at this stage was stirred continuously for one hour. The precipitate was then separated from the ethanol-acetone mixture by centrifugation, dissolved in 100 ml of distilled water, and heated at 100 C for 30 minutes. Insoluble particles appearing thereafter were removed by centrifugation and the rest of the solution was dialysed against several changes of distilled water at 4 C for 18 hours. The dialysed product was then freeze dried. The amount of ethanol soluble substance obtained from 100 ml of packed cells ranged between 700 and 900 mg. The method as outlined produced freeze-dried substances which were completely soluble in 0.85 per cent sodium chloride. A concentration of 200 mg of the freeze-dried substance per ml of saline (0.85% NaCl) was used.

Hemagglutination Inhibition Test

The specific serological activity of the various soluble substances was measured by the hemagglutination inhibition test using the procedure described by Kabat.[8] Suitable dilutions of known antisera containing predetermined hemagglutination units were mixed with serial dilutions of the substance to be tested. The highest dilution giving complete inhibition of hemagglutination was taken as the end point. Control tubes containing an equivalent amount of saline were set up simultaneously with the substance to be assayed. The various antisera used for the inhibition test were obtained from the diagnostic blood grouping laboratory of this institute where their serological specificities are routinely established against a panel of known red blood cells. The anti-Le^{bL} serum was obtained from a group O male Australian Aborigine[17] who possesses H substance in his saliva but not Le^a or Le^b. The addition of equal volumes of water soluble ABH substances did not reduce reactivity of the antibody or its specificity for Le^b positive red blood cells, indicating that this particular anti-Le^b can be classified as anti-Le^{bL}. The anti-P^k serum was obtained by absorbing anti-$P+P_1+P^k$ serum 'Sayed' with large volume of P_1 red blood cells at 37 C for one hour. The anti-P serum was donated by Dr. A. G. Matson of the War Memorial Blood Bank, Minneapolis. The amount of blood group activity present was indicated by the final saline dilution of ESS which was able to inhibit completely the optimal hemagglutination units of the corresponding antibody.

Results and Discussion

In a survey of the literature by Kabat,[8] it is shown that many different methods are available for the isolation and purification of soluble blood group substances from body fluids and tissues. Studies related to yields and possibly the presence of various specifications of blood group substances may therefore not be comparable when many different methods are employed.

We used a homogenizer to disrupt the red blood cells so that any biologically active blood group substance could be more effectively solubilized into ethanol. Acetone was then used to precipitate the carbohydrate and lipoprotein active groups from the ethanol. Boiling of the water-soluble precipitate ensured the removal of the lipoprotein groups, leaving only the carbohydrate groups in solution. Our method of isolating blood group active substances from the surface and cytoplasm of the red blood cells differs from the procedures cited by Kabat.[8] The physiochemical composition of the extracts will be the subject of a separate study.

The yield of soluble material obtained from 100 ml of packed cells was relatively uniform in relation to extracts recovered from the same donor's blood and similar to extracts from the red blood cells of different subjects (Table 1). When the extracts were dissolved in normal saline (0.85% Na Cl) at a concentration of 200 mg/ml and dilutions were tested against standardized antisera, no variations in the hemagglutination inhibition results were noted for the duplicate samples of bloods. This indicates that a real measure

Table 1. *Comparative Evaluation of ESS Recovered from Duplicate Samples of Blood*

Type of Blood	Coded Batch*	Weight of ESS Recovered from 100 ml of Packed Cells	Intensity of Inhibition Activity Demonstrated by ESS (200 mg/ml) Extracted from the Red Cells Using Optimal Hemagglutination Units of: Anti-A	Anti-H	Anti-P + P_1 + P^k
Group A_1	I	750 mg	256 (0.7 mg)	0	128 (1.5 mg)
	II	820 mg	256 (0.7 mg)	0	128 (1.5 mg)
Group O	III	720 mg	0	64 (3.1 mg)	256 (0.7 mg)
	IV	770 mg	0	64 (3.1 mg)	256 (0.7 mg)

*Preparations of the same bloods for 'blind' processing.

of uniformity in results can be expected when this extraction procedure is used for the comparative evaluation of substances in different types of blood.

Table 2 shows the inhibitory activity of various preparations of ESS obtained from human, monkey, and baboon red blood cells against standardized anti-A, anti-B, and anti-H. Group A_1 red blood cells possess as much soluble A substance as do $A_1H\uparrow$ cells, whereas A_2 red blood cells possess about a quarter of the amount of A substances. The difference in the amount of A substance observed between group A_1 and A_2 red blood cells is in agreement with the relative differences reported using A antigen site density measurements.[2] It was found that $A_1H\uparrow$ cells possess as much H substance as do group A_2 cells while group A_1 cells appear to lack these determinants. Red blood cells with a normal expression of A_1 and increased H are common in Southern African Negroes[1] and Indians.[11]

Group B red blood cells invariably contained a fair amount of B substances but, unlike blood group A_1 red blood cells, always showed some H activity. The highest amount of H activity was always recovered from group O cells and the findings suggest that these cells possess at least six times as much H substance as do group A_2 cells (Table 2). A surprising observation was the isolation of H substance from the single example of 'Bombay' Oh blood (Gov). This preparation of ESS not only inhibited an optimal concentration of the donor's own anti-H reagent but also other examples of anti-H of human and lectin origin. Race and Watkins[14] reported A-gene specific enzymes in the serum of this donor (Gov) and we also detected a small amount of serologically active A substance in the preparation of ESS obtained from the red blood cells.

Comparative studies suggest that the amount of H substance recovered from the 'Bombay' Oh cells is about the same as the amount from group A_2 cells. The significance of finding appreciable quantities of H substance in extracts of Oh cells when similar determinants cannot be detected on the cell membrane is still unclear. Poschmann, Fischer, Seidl, and Spielmann[13] recently found H determinants in a sample of 'Bombay' Oh blood after treatment of the red blood cells with an enzyme obtained from *Vibrio cholerae*. It was suggested that 'Bombay' cells may possess H determinants in the form of cryptantigens. The detection of H determinants on the red blood cells of individuals who also produce the corresponding antibody *in vivo* represents, at least in conventional circumstances, an autoimmune condition. The fact that *in vivo* red blood cell destruction has never been recognized in 'Bombay' bloods indicates that the H determinants are hidden and not accessible to the antibodies. It is clear that more extensive investigations are necessary to characterize the complexity of H substance in 'Bombay' Oh cells, including aspects of immunological concern.

From the point of view of serological specificities, it is known that man and nonhuman primates often differ with regard to

Table 2. *A Comparative Study of Anti-A, Anti-B and Anti-H Antibody Inhibition by Preparations of ESS Obtained from Various Human, Baboon, and Vervet Monkey Red Blood Cells*

Type of Blood	Number Tested	Average Intensity of Inhibition Activity Demonstrated by ESS (200 mg/ml) Extracted from Various Red Cells Using Optimal Hemagglutination Units of: Anti-A	Anti-B	Anti-H (Gov)
A_1	4	256* (0.7 mg)	0	0
A_1H †	3	256 (0.7 mg)	0	4 (5.0 mg)
A_2	3	64 (3.1 mg)	0	4 (5.0 mg)
B	4	0	128 (1.5 mg)	Less than 1
O	4	0	0	64 (3.1 mg)
Oh (Gov)	1	Less than 1	0	4 (5.0 mg)
Baboon pool 1 AH secretor	1	Less than 1	0	8 (2.5 mg)
Baboon pool 2 BH secretor	1	0	Less than 1	8 (2.5 mg)
Vervet pool 1 AH secretor	1	Less than 1	0	2 (100 mg)
Vervet pool 2 BH secretor	1	0	Less than 1	2 (100 mg)
Horse (control)	1	0	0	0

*Reciprocal of ESS dilution at which complete inhibition of agglutination for the corresponding antibody was found.

the distribution of the human type of ABO blood groups. For example, baboons and vervet monkeys do not express recognizable ABH antigens on their red blood cells while these antigens are almost always present on the red cells of man.[9,21] Evidence that 'Bombay' Oh cells, like the red blood cells of baboons and vervet monkeys, lack recognizable ABH antigens is remarkable because it indicates that there are exceptions to these rules. The isolation of hidden H substances from baboon and vervet monkey red blood cells, in addition to small amounts of A or B substance (the latter is dependent on whether the primate cells were obtained from saliva secretors of AH or BH substances) is perhaps the best additional evidence we have to show that a close serological relationship may exist between human Oh cells and primate cells. However, whereas 'Bombay' Oh individuals can produce anti-A, anti-B and anti-H, these antibodies are always absent in baboons and vervet monkeys. This immunological variability is undoubtedly influenced by the presence or absence of secretory AH or BH substances. It is also noteworthy that the ethanol soluble substance prepared by the same procedure from horse red cells consistently failed to react with the antisera used in this study.

It is generally accepted that Lewis blood group antigens are passively acquired by the red blood cells from the plasma.[6] In order that only the genetically predetermined and not the acquired forms of red blood cell antigens might be characterized, we subjected the cells to successive washings with large volumes of 0.15M Na Cl before ethanol fractionation. Such washing is known to dissociate autologous membrane-bound proteins.[5] Table 3 shows that the preparations of ESS obtained from the washed red blood cells still contained Le^a and Le^{bL} blood group substance. This indicates that the removal of membrane-bound plasma proteins did not dissociate the Lewis antigens. The absence of Lewis substances in preparation of ESS was found only in subjects who also lacked them in their secretions. This is in agreement with the facts known about the interrelationship between Lewis substances in the secretions and the red blood cells. The red blood cells of nonsecretors of ABH substances also possessed slightly more Le^a substance that did the red blood cells of secretors. The distribution of Le^{bL} did not show any marked variations whether the cells were obtained from secretors or nonsecretors of ABH substances.

It was found that baboons and vervet monkeys possess more Lewis substances,

Table 3. *A Comparative Study of Anti-Le^a and Anti-Le^{bL} Antibody Inhibition by Preparations of ESS Obtained from Various Human, Baboon, and Vervet Monkey Red Blood Cells*

Antigens on Red Cells	Specificities in Secretions	Number Tested	Average Intensity of Inhibition Activity Demonstrated by ESS (200 mg/ml) Extracted from Various Red Cells Using Optimal Hemagglutination Units Of: Anti-Le^a	Anti-Le^{bL}
A_1 Le^{a-b+}	AH Le^a Le^b	4	2* (100 mg)	2 (100 mg)
A_1 Le^{a+b-}	ns Le^a Le^b	3	8 (25 mg)	2 (100 mg)
O Le^{a-b+}	H Le^a Le^b	3	2 (100 mg)	2 (100 mg)
O Le^{a+b-}	ns Le^a Le^b	3	16 (12.5 mg)	2 (100 mg)
O Le^{a-b-}	H – –	2	0	0
Baboon pool 1	AH – Le^b	1	8 (25 mg)	32 (6.2 mg)
Baboon pool 2	BH – Le^b	1	8 (25 mg)	32 (6.2 mg)
Vervet pool 1	AH Le^a Le^b	1	8 (25 mg)	16 (2.5 mg)
Vervet pool 2	BH Le^a Le^b	1	8 (25 mg)	16 (2.5 mg)
Horse (control)	– – –	1	0	0

*Reciprocal of ESS dilution at which complete inhibition of agglutination for the corresponding antibody was found.

particularly Le^{bL}, it is significant that Le^a substance can be found on the red blood cells of baboons when they are known to lack these determinants in their secretions.[4,18] In this respect, the distribution of Le^a substance in baboons differs from that in man who not only has these substances in many secretions but also on the surface of many cell lines.[16]

The blood group activities for P, P_1, P^k and I are shown in Table 4. An unexpected observation was that baboon and vervet monkey cells have greater amounts of P^k substance than do human red blood cells. No variations were apparent for the distribution of P and P_1 determinants. Subjects with the very rare phenotype p ($P+P_1+P^k$ negative) did not have the expected P specificities in the ethanol soluble preparations.

The red blood cell phenotype I of normal adults is known to show a remarkable degree of variation in antigen strength, indicating that different amounts of I antigen can be present on the surface membrane.[3] We were unable to show that red blood cells with strong I activity possess more I substance than do the weaker examples of I positive

Table 4. *A Comparative Study of Anti-P, Anti-P_1, Anti-P^k, Anti-P + P_1 + P^k and Anti-I Antibody Inhibition by Preparations of ESS Obtained from Various Human, Baboon, and Vervet Monkey Red Blood Cells*

Antigens on Red Cells	Number Tested	Average Intensity of Inhibition Activity Demonstrated by ESS (200 mg/ml) Extracted from Various Red Cells Using Optimal Hemagglutination Units Of: Anti-P	Anti-P_1	Anti-P^k	Anti-P + P_1 + P^k	Anti-I
P I (++++)**	5	1* (200 mg)	0	64 (3.1 mg)	64 (3.1 mg)	1 (200 mg)
P,P_1 I (+)	8	1 (200 mg)	2 (100 mg)	64 (3.1 mg)	64 (3.1 mg)	1 (200 mg)
p (Tj^{a-}) I (++++)	2	0	0	0	0	1 (200 mg)
Baboon pool 1	1	2 (100 mg)	4 (50 mg)	1024 (0.15 mg)	512 (0.3 mg)	8 (25 mg)
Baboon pool 2	1	2 (100 mg)	4 (50 mg)	1024 (0.15 mg)	512 (0.3 mg)	8 (25 mg)
Vervet pool 1	1	2 (100 mg)	4 (50 mg)	512 (0.3 mg)	256 (0.7 mg)	8 (25 mg)
Vervet pool 2	1	2 (100 mg)	4 (50 mg)	512 (0.3 mg)	256 (0.7 mg)	8 (25 mg)
Horse (control)	1	0	0	0	0	0

*Reciprocal of ESS dilution at which complete inhibition of agglutination for the corresponding antibody was found.

**Predetermined serological characteristics of I antigen strength of the various donors.

red blood cells. Variations in agglutination reactions may therefore be due to a combination of other factors, *e.g.*, antigen-antibody binding affinity and density of protruding I antigen sites on the cell membranes.

When the various preparations of ESS were examined for their inhibitory activity against other varieties of red blood cell antibodies it was observed that Rh, MNSsU, Fy^a, Kell, Sd^a, Chido, and Bg^a antigens were not present in the extracts. The findings therefore indicate that the biochemical structure of these antigens may be dissimilar to that of the ABH, Lewis, I, and P red blood cell antigens.

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C. H. Vos, O.B.E., Ph.D., M. R. C. Path., Head, Experimental Immunology Unit, Natal Institute of Immunology, 149 Prince Street, Durban, South Africa 4001 (Reprint requests).

H. J. Downing, M. Agr. Sc., B.Ed., Ph.D., Head, Primate Centre, Natal Institute of Immunology, 149 Prince Street, Durban, South Africa 4001.

P. P. Moores, B.Sc., M. T. Officer-in-Charge, Blood Group Serology Unit, Natal Blood Transfusion Service, 149 Prince Street, Durban, South Africa 4001.

A. F. C. Mohideen, Dipl. Clin. Path. Dipl. Clin. Haematol., Senior Technician, Experimental Immunology Unit, Natal Institute of Immunology, 149 Prince Street, Durban, South Africa 4001.

Paper 33

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Hemagglutination Inhibition Studies of Water Soluble Blood Group Substances Recovered from the Erythrocytes of Classical Bombay O_h Subjects

G. H. Vos, and P. P. Moores

From the Natal Institute of Immunology, Durban, South Africa

Using ethanol and acetone fractionation to isolate soluble blood group substances from red blood cells, 'Bombay' O_h bloods were found to contain variable amounts of concealed H substance. The IgG variety of anti-H in 'Bombay' bloods has a greater affinity for these substances than the IgM variety of anti-H. Group O parents of 'Bombay' O_h subjects were found to have normal levels of H substance, indicating that individuals heterozygous for a recessive suppressor gene 'x' synthesize it normally. In the 'Bombay' family studied, Lewis determinants were abnormally expressed in two members. Lewis activity was detected in the soluble extracts of their red blood cells but not by the direct agglutination test. Further tests using known Le(a−b−) types are necessary to determine whether these findings are linked to the 'Bombay' O_h phenomenon.

In a previous study, we demonstrated that soluble substances related to blood groups A, B, H, Le^a, Le^{bL}, P, P_1, P^k, and I antigens could be isolated from the red blood cells by a process of ethanol and acetone fractionation.[19] Quantitative hemagglutination inhibition assays showed that the amount of substances recovered from the red blood cells did not always correlate with variations in antigen strength shown by the direct hemagglutination test. It was significant that blood group H substance could be isolated from the red blood cell membrane of a 'Bombay' O_h subject who synthesized anti-H which was neutralized by his own H substance *in vitro*. This indicates that the immune system did not have access to the concealed H determinants which had been present in the red blood cells since embryonic life.

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We have now examined the inhibitory reactivities of ethanol-soluble substances (ESS) from the red blood cells of seven healthy Indian 'Bombay' O_h subjects, and the parents and sibling of one of them. The reactions of these substances against the subject's own serum anti-H and other examples of human anti-H were studied. Biochemical studies are in progress to determine the presence of various sugars in the heat stable carbohydrate extracts from different red blood cells. It will be important to isolate the fucose unit that confers H specificity on 'Bombay' O_h bloods with concealed H substance.

Materials and Methods

Blood samples were obtained in ACD solution (acid-citrate-dextrose) from seven Indian 'Bombay' subjects, three group O controls and members of a family of whom the proposita (Nirmala M.) has the 'Bombay' O_h phenotype. Table I shows the family's blood groups and saliva secretor status. It was confirmed that the paternity of Nirmala is not in dispute and that her O_h phenotype is probably the result of recessive suppressor gene (xx) action.

Method of Obtaining Ethanol Soluble Blood Group Substances (ESS) from Red Cells and Defibrinated Plasma

Four hundred ml of 95 per cent purified ethanol was added to a 100 ml suspension of washed packed red blood cells. The mixture was placed in a 500 ml container designed for a Vertis blender and homogenized four times per hour for one minute at 60,000 rpm between the hours 9 am and 5 pm. After standing at room temperature overnight, the red blood cell debris was removed by centrifugation and the supernatant ethanol was placed in a two-liter glass beaker. Sodium chloride

Table 1. *Comparative Inhibition Activity of ESS (200 mg/ml) Extracted from Various Red Blood Cells for Optimal Hemagglutination Units of IgM and IgG Reactive Anti-H Reagents Produced by 'Bombay' Oh Subjects*

Subjects	Antigens on Red Cells	Specificities in Secretions	Optimal Hemagglutination Units of Saline Agglutinating Anti-H of 'Bombay' Subjects						Optimal Hemagglutination Units of 2-ME Treated Anti-H of 'Bombay' Subjects (Enzyme Test)					
			Gov.	Naid.	Soo.	Bal.	Lee.	Rug.	Gov.	Naid.	Soo.	Bal.	Lee.	Rug.
M. Gov.	O_h Le^{a+b-}	ns Le^a –	8*	2	4	0	0	0	4	4	8	4	4	8
G. Naid.	O_h Le^{a+b-}	ns Le^a –	4	2	4	0	0	0	4	4	4	2	4	2
N. Soo.	O_h Le^{a+b-}	ns Le^a –	0	0	0	0	0	0	8	4	4	8	4	4
G. Bal.	O_h Le^{a+b-}	ns Le^a –	4	0	2	0	0	0	4	4	2	4	4	4
D. Lee.	O_h Le^{a+b-}	ns Le^a –	0	0	2	0	0	0	4	4	8	4	2	2
D. Rug.	O_h Le^{a+b-}	ns Le^a –	0	0	0	0	0	0	8	4	8	4	4	8
Mr Moon.	O Le^{a-b-}	H –	64	128	64	128	128	128	128	128	128	64	128	128
Mrs Moon.	O Le^{a+b-}	ns Le^a –	8	4	4	8	4	8	8	16	8	8	4	8
Nirm. Moon.	O_h Le^{a+b-}	ns Le^a –	0	0	0	0	0	0	4	4	4	8	2	4
Pris. Moon.	O Le^{a-b-}	ns – –	4	2	4	8	4	4	8	4	4	4	8	8
G.V.	O Le^{a-b+}	H Le^a Le^{bL}	128	128	64	128	64	128	64	128	128	128	64	128
R.N.	O Le^{a+b-}	ns Le^a Le^{bL}	8	4	2	4	8	4	8	8	4	8	4	8
R.D.	O Le^{a-b-}	H – –	128	128	128	128	64	128	128	128	128	128	128	128

*Reciprocal of ESS dilution at which complete inhibition of agglutination for the corresponding anti-H reagents was found.

was added and the undissolved salt removed. The beaker containing the ethanol was then placed on a mechanical stirrer in a 4 C refrigerator. Four volumes (1.6 liters) of cooled acetone was slowly added to the ethanol and the white precipitate which formed at this stage was stirred continuously for one hour. The precipitate was then separated from the ethanol-acetone mixture by centrifugation, dissolved in 100 ml of distilled water and heated at 100 C for 30 minutes. Insoluble particles appearing thereafter were removed by centrifugation and the rest of the solution was dialyzed against distilled water at 4 C for 18 hours. The dialyzed product was then freeze-dried. This method produced freeze-dried substances that were completely soluble in 0.85 per cent sodium chloride solution. A concentration of 200 mg of substance per ml of solution was used for the investigations considered here.

Hemagglutination Inhibition Test

The specific serological activity of the various red blood cell soluble substances was measured by the hemagglutination inhibition test described by Kabat.[8] To 0.2 ml amounts of suitable dilutions of known antisera (anti-A, anti-P, and anti-Le , etc.) containing predetermined hemagglutination units were added serial dilutions of the substance to be tested. The highest dilution giving complete inhibition of hemagglutination was taken as the end point. For each antibody system, control tubes containing an equivalent amount of saline were set up simultaneously with the substance to be assayed. The various antisera used were obtained from the diagnostic blood grouping laboratory of this institute where their serological specificities are routinely established against a panel of known red blood cells. The amount of blood group activity present was indicated by the final saline dilution of ESS which was able to inhibit completely the optimal hemagglutination units of the corresponding antibody.

Serological Methods

The Saline Test. Saline agglutinating antibody activity was measured by placing one volume of known antiserum on a translucent glass tile to which was added an equal volume of a 5 per cent saline suspension of washed red blood cells. The glass tile was then placed in a moist chamber for one hour at the optimal temperature for the corresponding antibody. The results were read with a 5× magnification head-fitting eyepiece.

The Ficin Test. A solution was prepared of 0.25 per cent ficin (Calbiochem C grade fig latex) using Bacto hemagglutination buffer as the suspending fluid. An equal volume of the prepared enzyme solution was added to a 5 per cent suspension of saline washed test cells. The mixture was allowed to stand at room temperature for 10 minutes. The cells were then washed three times in normal saline. From this point onward, the method of determining antibody activity was the same as for the saline test.

Mercaptoethanol Test. The serum was treated by adding one volume of 0.2M 2-mercaptoethanol in saline to one volume of heat inactivated serum. The mixture was incubated overnight at 4 C and

tested the following morning by the two procedures described above.

Results

Table 1 shows the inhibition activity of heat-stable carbohydrates extracted from normal group O red blood cells and from 'Bombay' O_h cells examined against anti-H isoantibodies from 'Bombay' subjects. Lectin and protectin anti-H were avoided because the spectrum of their reactivities is not absolutely identical.[2] Previous studies showed that almost all 'Bombay' subjects produce anti-H as a mixture of IgM and IgG isoantibodies. The IgM isoantibodies possess intense agglutinating activity for group O cells suspended in saline, and the IgG isoantibodies readily agglutinate enzyme treated cells after the serum has been treated with mercaptoethanol. With these two varieties of anti-H, we found that the extracts from normal group O cells inhibited optimal concentrations of both the saline (IgM) and enzyme (IgG) agglutinating anti-H reagents. However, the extracts from 'Bombay' O_h cells had a greater affinity for the IgG variety of anti-H. This suggests that differences may exist between normal group O and 'Bombay' O_h bloods with respect to the chemical configuration of the heat-stable carbohydrates that carry the H determinant groups. It is also evident that group O subjects who secrete H substance in their saliva possess greater concentrations of H determinants on their red blood cells than do nonsecretors. It was surprising to find that the ethanol soluble substances from 'Bombay' O_h subjects contained as much soluble H substances as those extracted from normal group O nonsecretors.

According to the biosynthetic pathways suggested by Watkins and Morgan[20] and Ceppellini,[4] 'Bombay' O_h subjects are homozygous for the recessive suppressor gene that prevents the formation of H substance from precursor substance. This theory also presupposes that the parents of 'Bombay' subjects are heterozygous for the suppressor gene, implying that they might produce less H substance than do normal group O subjects. We examined this hypothesis by determining the amount of H substance produced by the parents of a 'Bombay' O_h person. Table 1 shows that the red blood cells of both parents produced about the same amount of H substance as did the red blood cells of normal group O secretors and nonsecretor controls. It would therefore appear that individuals heterozygous for the recessive suppressor gene (x) produce enough precursor substance for the formation of normal levels of H substance.

Current knowledge suggests that the synthesis of Lewis and ABH determinants is regulated by independent genes operating on a common substrate and that this type of linkage may be responsible for complex phenotypic interaction between the two blood group systems. In a previous study,[19] we found that the absence of Lewis substance in preparations of red blood cell extracts was associated with the absence of Lewis expression in the saliva. This type of correlation was observed again in the present study in one example of a Lewis nonsecretor (R. D., Table 2), but not in two others who lacked the Lewis factor in their secretions. In these two subjects, a small amount of Le^a and Le^{bL} substance was detected in their red blood cell extracts although they are known to lack the corresponding factors in their saliva and on the surface membrane of their red blood cells. Biochemical investigations by Hakomori and Strycharz[7] suggest that Lewis specificity is not only acquired as a glycoprotein from the plasma but that it may also exist within the cell membrane as a glycolipid. This could explain why we were unable to recognize Lewis antigen on the surface of intact cells. On the other hand, our inability to recognize some intermediate form of Lewis antigen on the surface of Le(a−b−) red blood cells may also be influenced by the type of antisera used. Lodge, *et al.*[12] showed that certain rare Lewis antibodies do react with group O and O_hLe(a−b−) red blood cells. We also found that nonsecretors of H substance with Le^a substance in their saliva had more alcohol soluble heat-stable Le^a on the red blood cells than did those with H, Le^a, and Le^{bL} substances in their secretions.

I antigen activity is known to be present in human saliva, milk, and red blood cell stroma.[6,13] It has also been reported that 'Bombay' O_h subjects possess more I antigen activity on their red cells than do normal group O subjects.[5,14] Using several examples of anti-I reagents which strongly sensitize intact O_h cells by the direct agglutination test, we were unable to show that the soluble extracts obtained from 'Bombay' O_h bloods had more I substance than did those from other types of blood (Table 2). Variations in direct agglutination reactions therefore need not necessarily reflect quantitative differences in soluble I antigen strength.

Plant extracts which agglutinate erythrocytes and other cells are referred to as phytohemagglutinins. Tillack, *et al.*[18] reported that the binding activity of phytohemagglutinins is closely associated with ABO blood groups. Using chromatographic separation procedures, Zuckerman, *et al.*[21] found that the phytohemagglutinin activity of group AB bloods always separated with the group A and not the group B

Table 2. *Comparative Inhibition Activity of ESS (200 mg/ml) Extracted from Various Red Blood Cells for Optimal Hemagglutination Units of Anti-Le^a, Anti-Le^{bL}, anti-I and Phytohemagglutinin*

Subjects	Antigens on Red Cells	Specificities in Secretions	Inhibition Activity Demonstrated by ESS from Various Red Cells Against Optimal Hemagglutination Units of Anti-: Le^a (143)	Le^a (132)	Le^a (68)	Le^{bL} (183)	Le^{bL} (29)	Le^{bL} (78)	I (100)	I (136)	PHA (Wellcome)
M. Gov.	O_h Le^{a+b-}	ns Le^a —	4*	8	16	1	2	4	2	4	0
G. Naid.	O_h Le^{a+b-}	ns Le^a —	8	16	32	2	4	4	2	4	0
N. Soo.	O_h Le^{a+b-}	ns Le^a —	8	16	64	2	4	8	2	4	0
G. Bal.	O_h Le^{a+b-}	ns Le^a —	16	32	64	1	4	2	2	4	0
D. Lee.	O_h Le^{a+b-}	ns Le^a —	32	32	64	0	2	4	2	4	0
D. Rug.	O_h Le^{a+b-}	ns Le^a —	32	32	32	0	2	2	2	4	0
Mr Moon.	O Le^{a-b-}	H — —	2	4	4	2	4	8	2	4	0
Mrs Moon.	O Le^{a+b-}	ns Le^a —	32	16	32	2	4	4	2	4	0
Nirm. Moon.	O_h Le^{a+b-}	ns Le^a —	32	32	32	2	8	4	2	4	0
Pris. Moon.	O Le^{a-b-}	ns — —	4	4	8	2	8	8	2	4	0
G.V.	O Le^{a-b+}	H Le^a Le^{bL}	8	4	16	8	64	32	2	4	0
R.N.	O Le^{a+b-}	ns Le^a Le^{bL}	32	32	64	2	8	4	2	4	0
R.D.	O Le^{a-b-}	H — —	0	0	0	0	0	0	2	4	0

*Reciprocal of ESS dilution at which complete inhibition of agglutination for the corresponding antibodies was found.

determinant. We could not demonstrate phytohemagglutinin activity in the ethanol-acetone extracts of normal group O and 'Bombay' O_h red blood cells (Table 2) although the intact cells of the same subjects were always strongly agglutinated by the plant extracts. This indicates that the phytohemagglutinin receptor on the red blood cell membrane is not heat resistant and that it is not closely linked to the H determinant of the ABO blood groups.

Discussion

Although many aspects of membrane serology and biochemistry can be discussed from the results obtained in this study, we are at the moment most concerned with the question of how nature has concealed the H antigen on 'Bombay' red blood cells from the destructive effects of the subjects' own antibodies *in vivo*. A review of the various types of aberrant A, B, and O blood groups and the development of isoantibodies produced by them may be a useful beginning.

Since the discovery of the first 'Bombay' O_h bloods by Bhende, *et al.*,[1] it has become clear that there are at least two phenotypically distinct 'Bombay' bloods. One includes 'Bombay' O_h subjects whose red blood cells do not absorb anti-A, anti-B, or anti-H antibodies (classical type). The other comprises those whose red blood cells are able to absorb ABH antibodies, although these do not agglutinate them.[5,10,11] The classical type is called O_h, the others O_h^A, O_h^B and O_h^H. Neither kind of subject secretes ABH substances. Fundamental differences in isoantibody formation have been observed between the two kinds of 'Bombay' subjects, indicating that the presence of even small amounts of A or B determinants on their red blood cells can have a profound effect on their ability to make the corresponding ABH isoantibodies. Dzierzkowa-Borodej, *et al.*[5] demonstrated that O_h^A subjects produce stronger anti-B than anti-A or anti-H isoagglutinins. In the classical O_h subjects reported by Moores, *et al.*,[14] no significant differences in anti-A, anti-B, or anti-H titers were observed.

The formation of weaker anti-A isoantibodies by O_h^A subjects implies that the antigenicity of the A determinant on the red blood cell membrane is different from the specificity detected by the isoantibody. If this were not so, then the antibody would have to be accepted as an autoantibody. Since red blood cell survival studies using ^{51}Cr-labeled autologous cells did not indicate that 'Bombay' O_h subjects suffer from autoim-

mune hemolytic anemia,[15] it can be accepted that the isoantibodies are not directed against their own intact red blood cells *in vivo*. The observation that anti-H isoantibodies from classical 'Bombay' O_h subjects can be inhibited *in vitro* by H determinants isolated from their own red blood cells contrasts with the *in vivo* behavior of the isoantibody and calls for clarification of the structural configuration of the H determinants on 'Bombay' O_h red blood cells. Our findings indicate that fundamental differences in the carbohydrate structure of cell membranes must exist between normal group O and 'Bombay' O_h subjects.

The existence of strong anti-H isoantibodies in the serum of 'Bombay' O_h subjects also implies nonrecognition of the concealed H determinants (O_h^A subjects produce low values of anti-A isoantibodies) and a total lack of intrinsic immunological tolerance toward them. Their complete absence at the surface of the cell membrane, where immune surveillance is believed to distinguish self from not-self,[3] enables O_h subjects to make antibodies to H determinants as a result of nonovert immunization by natural microflora.

If individuals with aberrant ABH expression on their red blood cells secrete the corresponding blood group substances in their saliva, it can be expected that this will affect the synthesis of anti-A, anti-B, or anti-H isoantibodies. The finding of bloods with O_m^h,[17] and B_h[9] phenotypes are good examples. O_m^h individuals lack cellular H and serum anti-H but possess normal quantities of H in their saliva. The red blood cells of B_h subjects do not react with anti-H, are practically inagglutinable by various anti-B reagents, and fail to produce antibodies to blood factors B and H because the corresponding antigens are present in their saliva. A similar situation appears to exist in baboons and vervet monkeys.[19] They possess the human variety of ABH substances in their secretions and appear to express the O_h^A or O_h^b blood type on their cells, a combination that prevents the development of natural anti-A, anti-B, and anti-H isoantibodies.

The findings show the restricted process under which the immune system operates when the corresponding substances are present in a soluble state. This type of restriction does not seem to operate when the antigenic determinants are not accessible to the immune system as in classical 'Bombay' O_h subjects.

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G. H. Vos, O.B.E., Ph.D., D.Sc., M.R.C. Path., Head, Experimental Immunology Unit, Natal Institute of Immunology, 149 Prince Street, Durban, South Africa, 4001.

P. P. Moores, B.Sc., M.T. Officer-in-Charge, Blood Group Serology Unit, Natal Blood Transfusion Service, 149 Prince Street, Durban, South Africa, 4001.

Paper 27

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Some Observations on "Bombay" Bloods, with Comments on Evidence for the Existence of Two Different O_h Phenotypes

P. P. MOORES, P. D. ISSITT, B. G. PAVONE, AND B. G. MCKEEVER

From the Natal Blood Transfusion Service, Durban, South Africa and the Paul I. Hoxworth Blood Center University of Cincinnati, Cincinnati, Ohio

Bloods from three individuals, one each of the phenotypes O_h^A, O_h^B and O_h^O have been studied. The work of Dzierzkowa-Borodej, *et al.*[10] was confirmed when it was shown that all three samples of O_h red blood cells had increased I antigen strength. The i, Sd^a, Le^a and Le^x antigens were not found to be increased. Attempts were made to adsorb and elute anti-A, anti-B and anti-A,B with the O_h red blood cells, using sera that contained high titered anti-I antibodies. This was done in the belief that previously reported positive results in such tests might be due to the high level of I on the O_h red blood cells, anti-I in the sera containing the ABO antibodies, and the Matuhasi-Ogata phenomenon. However, in no instance were we able to adsorb an ABO antibody onto the O_h red blood cells. Contrary to the report of others[10] the titers of anti-A, anti-B and anti-H in the sera of the three O_h individuals studied did not differ significantly.

We suggest that the evidence from our findings and the work of others is sufficient to show that at least two forms of the O_h phenotype exist: one representing total suppression of H, A, and B antigens, and the other marked but not total suppression, with partial inhibition of antibody production.

SINCE the original report of the "Bombay" phenotype by Bhende, *et al.*,[4] the accuracy of a theory proposed by Ceppellini, *et al.*[7] and by Watkins and Morgan,[35] that provided a genetic background for this phenotype, has been repeatedly confirmed by the family studies of Levine, *et al.*[20] and others.[2,3,6,14,19,36] The theory states that in the absence of a very common gene *H*, no H substance is produced. As a result, the *A* and *B* genes, that act by controlling the addition of immunodominant sugars to the H precursor chain, are not expressed. "Bombay" (O_h) individuals, genetically *hh* at the *Hh* locus, therefore have normal genes at the *ABO* locus and are able to pass these to their offspring where, in the presence of an *H* gene provided by the other parent, they are expressed in the usual way. Watkins, *et al.*[36] have recently shown also that the *H* gene specified alpha-2-L-fucosyltransferase is not present in the serum of O_h persons, but those who are O_h^A have *A* gene specified alpha-N-acetylgalactosaminyltransferase, and those who are O_h^B have *B* gene specified alpha-D-galactosyltransferase.

Although it is universally accepted that the quantities of A and B antigens on O_h red blood cells are insufficient to support agglutination of those red blood cells by anti-A, anti-B, and anti-A,B, some workers[10,13,19,20] have found that O_h^A red blood cells will adsorb and elute anti-A while O_h^B red blood cells will adsorb and elute anti-B. Although others[30] have not been able to confirm these observations with all O_h^A and O_h^B red blood cells, it has been generally accepted that the positive adsorption-elution experiments indicate that these red blood cells carry small amounts of A and B antigens respectively.[16,30]

In 1972, Dzierzkowa-Borodej, *et al.*[10] reported studies on a family in which two brothers were of the phenotype O_h^A. The red blood cells of these two individuals were shown capable of adsorbing and eluting anti-A and anti-H and in addition it was found that their sera contained less anti-A

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than anti-B. Thus, it seemed possible that the phenotypes O_h^A and O_h^B both might be recognizable serologically.[13] The red blood cells seemed to carry trace amounts of ABH antigens, the presence of which seemed to partially inhibit antibody production. These workers[10] also reported that O_h red blood cells had increased I antigen that was demonstrable with most, but not all, examples of anti-I.

Samples of red blood cells and plasma from three "Bombay" phenotype individuals, known from family studies and from the serum transferase enzyme tests of Watkins, *et al.*[36] to be O_h^A, O_h^B, and O_h^O, were available and were examined by us for red blood cell levels of the antigens I, i, Sd^a, Le^a, and Le^x. We also titrated the anti-A, anti-B, and anti-H antibodies in the serum of each of these individuals. In adsorption and elution experiments, we used anti-A, anti-B, and anti-A,B. In these studies, some sera contained anti-I and some sera had the anti-I removed by adsorption. If the ABO antibodies containing anti-I had been adsorbed and eluted by the O_h red blood cells (with high levels of I antigen) while ABO antibodies from which anti-I had been removed by adsorption were not adsorbed to similar cells, it would have been shown that the recovery of ABO antibodies from O_h red blood cells was not necessarily an indication of the presence of A or B antigens, as it could also be explained by the Matuhasi-Ogata phenomenon.[1,23,26,27]

Although we confirmed the increase of I on O_h red blood cells, we were not able to adsorb, or recover by elution, anti-A, anti-B, or anti-A,B from the red blood cells of any of these three O_h individuals. Further, we found no significant differences in the ABO agglutinin levels in the sera of the three persons. The results of our experiments are reported herein and, because we were unable to find any evidence of A, B, or H antigens on the O_h red blood cells we studied, we postulate that there are at least two different expressions of the phenotype that has been described as O_h.

Materials and Methods

The samples of blood from the three adult O_h donors and from a suitable group O adult control donor were collected into ACD solution in Durban on the same day and under the same conditions. The O_h samples were then divided into two portions and one portion of each was sent by air to Cincinnati where suitable group O, A, and B red blood cells, collected on the same date, were used as controls. The tests in both laboratories were completed within two weeks of the date of collection of the samples and there was no indication in Cincinnati that the O_h bloods had been adversely affected in any way by shipment. Since attempts were to be made in Cincinnati to identify the O_h^A, O_h^B, and O_h^O samples by serological studies, the bloods were labelled 1, 2, and 3 in Durban and their exact identity was not known in Cincinnati until all tests had been completed. The O_h^A, O_h^B, and O_h^O phenotype of each donor had been established by prior family study and serum transferase enzyme tests.[36] For the antibody titrations the ACD plasmas were converted to sera by recalcification.[16] Although this method results in some dilution, this was of no consequence since the object of the tests was to compare the anti-A, anti-B, and anti-H levels in each individual serum.

Titrations in both laboratories were performed by standard techniques.[16] All red blood cell samples were washed at least twice prior to testing, then resuspended in 0.85 per cent saline (final red blood cell suspension 3 to 4 per cent in Cincinnati, 5 to 6 per cent in Durban). Where necessary, the red blood cells were pretreated with ficin (0.1 per cent in Cincinnati, 0.25 per cent in Durban) or a proteolytic enzyme solution (papain in Cincinnati, bromelin in Durban) was added directly to the cell-serum mixtures. Titrations were incubated at 22 C or 4 C dependent on the antibody being used, for up to 60 minutes before being read.

Adsorption studies utilized a volume of serum mixed with an equal volume of washed packed red blood cells. The mixtures were incubated (60 minutes in Cincinnati, overnight in Durban) at the optimum reaction temperature of the antibody involved. Eluates were made by the heat method of Landsteiner and Miller.[18] This has been found to be more efficient than other techniques for the recovery of cold-reacting antibodies, especially those of the ABO system.[17]

The anti-I sera used in the study were from a variety of sources. They included potent autoagglutinins from patients with cold hemagglutinin disease, autoagglutinins from patients and blood donors with no evidence of *in vivo* hemolysis, and alloantibodies from I-negative indi-

viduals. Details of these antibodies are shown in Table 1. Other reagents used included an anti-i from a group A_1B Caucasian, an anti-Sd^a from a group A_1B Negro, an anti-Le^a from an Le (a-b-) Negro, two anti-Le^x antibodies from Le (a-b-) Negroes and a variety of antibodies directed against high incidence and low incidence antigens. All of these sera were used with suitable positive and negative control red blood cell samples when the O_h red blood cells were tested.

The anti-A, anti-B, and anti-A,B antibodies used in Durban were selected for their high titers and immune character that had previously been determined by a 2-mercaptoethanol and secretor saliva test.[25] Those used in Cincinnati were selected because they contained high levels of anti-I in addition to appreciable amounts of anti-A or anti-B, and were divided into two aliquots before the studies were undertaken. One aliquot of each serum was used with the anti-I still present, the other was adsorbed with ficin-treated group O red blood cells until all demonstrable anti-I had been removed.[18] Tests following adsorption showed that removal of the anti-I had not significantly altered the ABO antibody levels.

A_1, B, and O red blood cells used to titrate the serum antibodies of the O_h individuals were freshly drawn from normal donors, washed three times, and resuspended in 0.85 per cent saline to a concentration of 3 to 4 per cent for use.

Results

Titration studies with many examples of anti-I from different sources showed that O_h red blood cells frequently react more strongly than red blood cells from non-Bombay adults. The results in Table 1 are expressed as titer scores.[29] It was also found that the enhanced I on O_h red blood cells resulted in a higher titer end point with most of the sera that recognized the increase of I. Of the 26 anti-I sera used, 16 revealed a marked increase of I on O_h red blood cells; three a less marked but still obvious increase, and the remaining seven either no increase or a lower titer score than with the non-Bombay con-

Table 1. *Titration Scores of Anti-I Sera with Three O_h and Many Control Red Blood Cells*

		Titer Scores With			
Anti-I Serum	Source*	O_h^O	O_h^A	O_h^B	Non-O_h Average of Many
PPM 136	D Allo-i†	86	77	86	67
PPM56/BRU72	D Allo-i	76	77	80	57
M 261	D Auto-norm‡	85	85	86	55
PPM 18	D Allo-i	35	35	47	26
PPM 117	D Allo-i	75	67	75	53
PPM 100	D Auto-norm	82	67	77	44
PPM 73	D Allo-i	55	41	46	30
P Zulu	D Allo-i	43	46	46	18
M 309	D Allo-i	35	35	38	18
M 370	D Allo-i	93	89	93	51
Mafuya	D Allo-i	79	79	79	57
R # 1	C Auto-norm	43	37	33	13
R # 2	C Auto-norm	40	38	41	30
R # 8	C Auto-CHD§	116	112	114	74
UC # 1	C Allo-i	10	25	21	32
UC # 4	C Auto-norm	32	36	40	35
UC # 5	C Auto-norm	32	33	31	34
UC # 7	C Allo-i	11	6	12	0
06753	C Auto-norm	24	17	25	23
09571	C Auto-norm	9	10	13	2
36542	C Auto-norm	80	80	77	58
06645	C Auto-norm	50	62	63	53
15908	C Auto-norm	56	56	54	44
09727	C Auto-norm	12	19	14	13
R # 3	C Allo-i	52	41	40	57
UC # 9	C Auto-CHD	140	133	140	111

*D = Durban, C = Cincinnati.
†Allo-i = Anti-I from an I-negative individual.
‡Auto-norm = Anti-I from an I-positive individual with no evidence of *in vivo* red cell destruction.
§Auto-CHD = Anti-I from an I-positive individual with cold hemagglutinin disease.

Table 2. *Titration Studies with Red Cells of Three O_h Individuals and Various Antibodies*

	Titer Score and Antibody				
				Anti-Le^{x}**	
Red Cells	Anti-i	Anti-Sd^{a}*	Anti-Le^{a}	1	2
O_h^{O}	14	32	69	83	66
O_h^{A}	24	31	70	81	61
O_h^{B}	28	33	72	83	71
Positive control	72†	30	71	83§	71§
Negative control	27‡	0	0	0	0

*Microscopic readings.
†Cord i red cells.
‡Adult I red cells.
§4 Adult Le(a+b−) red cells.
**Both reactive with cord blood red cells.

trol red blood cells. The possible reasons for the differences observed will be discussed later.

The titer score results of tests using the three O_h red blood cell samples and anti-i, anti-Sd^{a}, anti-Le^{a}, and anti-Le^{x} were essentially the same as the adult non-Bombay control red blood cells and are shown in Table 2. It should be noted that the anti-i reacted with both adult and cord red blood cell samples and that the reactions of the O_h red blood cells paralleled the adult samples, being less than those of the cord red blood cell samples.

In adsorption and elution tests, three anti-A sera (two with titers above 256, one with a titer of 128), six anti-B sera (all with titers above 256) and two anti-A,B sera (both with titers above 256 with A_1 and B red blood cells) were used. Eight of the 11 sera were tested prior to, and following removal of their anti-I. These sera contained anti-I antibodies that ranged in titer from 32 to 512. No evidence of adsorption of any of the ABO antibodies to any of the O_h red blood cells was obtained. The sera containing the ABO antibodies were titrated and there was no reduction of titer of an ABO antibody in any of them as a result of adsorption of the sera with the O_h red blood cells. Eluates made from the O_h red blood cells used in the adsorption tests were shown not to contain ABO antibodies when tested against A_1 and B red blood cells by agglutination tests at 4 C, agglutination tests using ficin and bromelin to enhance the sensitivity of the tests, and antiglobulin tests following incubation at 37 C. Some of the eluates did contain anti-I. Normal A_1 and B red blood cell samples were used in parallel adsorption and elution experiments with the same ABO antibodies and the results demonstrated that all adsorption, elution, and antibody detection tests were efficacious.

The serum samples from the three O_h donors were titrated against A_1, B, and O red blood cells. As can be seen from the results shown in Table 3, no significant differences in levels of anti-A, anti-B, and anti-H were seen in any single serum. The guesses made in Cincinnati of the genetic status of the coded samples, as gauged from the results of these titrations, are also shown in Table 3. It can be seen that not only were almost all of the guesses wrong, but that the serum antibody titers are in no way correlated with the O_h^{A}, O_h^{B}, or O_h^{O} status of the bloods tested.

The red blood cells of the three O_h individuals were shown to carry the high incidence antigens Vel; At^{a}; Yt^{a}; Jr^{a}; Gn^{a}; Di^{b}; Gerbich; Gy^{a}, Holley; Yk^{a}; and Chido, and to lack the low incidence antigens Di^{a}; Ls^{a}; Wb; Bu^{a}; Be^{a}; and Sk^{a}.

Discussion

Our finding that most anti-I sera react more strongly with O_h than with non-

Table 3. *Titrations of Anti-A, Anti-B, and Anti-H in the Sera of Three O_h Individuals*

Sample Number	Titrated Against Red Cells of Group	Tests at R.T.		Tests at 4 C		Cincinnati Guess at Phenotype	Actual Phenotype
		Titer	Score	Titer	Score		
1	A_1	128	79	128	81	O_h^{B}	O_h^{O}
	B	128	75	128	75		
	O	128	77	256	78		
2	A_1	64	57	64	63	O_h^{O} or O_h^{A}	O_h^{A}
	B	64	63	64	64		
	O	32	52	64	54		
3	A_1	128	71	128	73	O_h^{O}	O_h^{B}
	B	128	78	128	73		
	O	64	55	64	50		

Bombay adult red blood cells confirms that of Dzierzkowa-Borodej, *et al.*[10] Those workers suggested that the anti-I sera that they call anti-I^s, and which are inhibitable by human milk and saliva, are the ones most likely to detect the enhanced I on O_h red blood cells. However, it is apparent from our results in Table 1 that there is little, if any, correlation between the source of an anti-I and its ability to detect the enhanced I on O_h red blood cells.

It is not surprising that the I antigen is present in greater quantities on O_h than on normal red blood cells. There is good evidence[11,12] that I antigenic determinants are located on the mucopolysaccharide chains to which the H, A, and B determinants are eventually added. Thus, it is easy to visualize that the H, A, and B determinants normally "cover" some I structures and that when they are absent (as in O_h) more I is left exposed. Feizi, *et al.*[12] suggest that anti-I reagents are heterogeneous and have supplied evidence for at least six different specificities. In view of their findings, the anti-I sera that give enhanced reactions with O_h red blood cells may belong to the type capable of detecting I antigenic determinants that are normally "hidden" or "covered" by H, A, and/or B antigens. Those anti-I reagents that do not differentiate between O_h and non-O_h red blood cells may detect I antigenic determinants that are not normally "covered" by H, A, or B. The P blood group antigens are also believed to be associated with the mucopolysaccharide chains that carry I, H, A, and B,[28,33,34] and it is worth reporting here that using a single example of Tj(a-) red blood cells and two anti-I sera, no enhancement of I in the absence of Tj^a was noticed.[15]

Since not all O_h red blood cell samples described in the literature appear capable of adsorbing, and yielding on elution ABO system antibodies, corresponding to the ABO antigen suppressed, we wondered if those that had yielded anti-A, anti-B and/or anti-H, had done so because of their high levels of I antigen; the presence of anti-I in the sera containing the ABO antibody; and the Matuhasi-Ogata phenomenon.[1,26,27] Although it is now well established[5] that the nonspecific uptake of an antibody by red blood cells is not totally dependent on the presence of a specific antigen-antibody complex, it has been shown[23,24] that there is a greater chance of demonstrating the nonspecifically bound antibody in an eluate that contains a previously specifically bound antibody than in one that does not. However, although the O_h red blood cells tested here had enhanced I antigens and some of the anti-A and anti-B sera contained potent anti-I antibodies (titers up to 512), we obtained no positive eluates from the O_h red blood cells.

The evidence in the literature of successful adsorption and elution of ABO antibodies from O_h red blood cells by some workers,[10,13,19,20] but not by others,[30] and our own results reported here shows that O_h persons are almost certainly divisible into at least two categories. These comprise one group of persons whose red blood cell phenotype represents total suppression of H, A, and B antigens so that their red blood cells will not adsorb or give up ABO antibodies on elution; and a second group in whom marked but not total suppression of H, A, and B antigens occurs to a degree that prevents agglutination of the red blood cells by ABO antibodies but results in positive adsorption and elution experiments. The studies of others[10] and ourselves on the anti-A, anti-B, and anti-H titers in the sera of O_h persons support our view in that those with red blood cells able to adsorb ABO antibodies the titers of the alloantibodies were unequal, while in those with red blood cells that did not adsorb ABO antibodies the titers were similar to each other. It would be expected that the presence of trace amounts of an antigen would at least partially inhibit production of the corresponding antibody.

The difference in genetic background that leads to total, as opposed to almost total, suppression of H, A, and B could be one of several possibilities. One is that instead of the postulated *H* and *h* genes at the *Hh* locus there are a series of alleles that differ

slightly in their functional abilities. The genotype *hh* may result in no production of H, while an allele similar but not identical to *h* may allow small quantities of H (and consequently A and/or B) to be made. A second possibility is that both types of O_h represent the same *hh* genotype and that the genes behave a little differently in nonidentical environments. A similar idea for the $X^o r$ and X^Q genes of the Rhesus system has been postulated by Chown, *et al.*[8] A third possibility is that *H* and *h* (or perhaps just *H*) are genes of structure that are modified in their actions by genes of control at a different locus. All three possibilities provide hope of eventually uniting, in one genetic pathway, not only the two types of O_h, but also the A_h and B_h phenotypes,[21] O_m^h and A_m^h,[31] and possibly the *Yy* genes[37] that affect the expression of A as well. Even without precise knowledge of the genetic backgrounds that result in the two different types of O_h, it appears that the situation creates a phenotypic and serological analogy between the ABO and Rhesus blood group systems. In the $X^o r/X^o r$ type of Rh_{null}, no CDE antigens are made in spite of the presence of normal *CDE* genes. In the type of O_h individual studied here, the *hh* genes seem analogous to the $X^o r$ genes in that they block the actions of normal *ABO* genes present, just as $X^o r$ block normal *CDE* genes. The O_h individuals whose red blood cells adsorb and elute anti-A and/or anti-B can be thought of as serologically analogous to the type of Rh_{null} individual first described by Stevenson, *et al.*[32] whose red blood cells type as Rh_{null} but are able to adsorb, and yield on elution, certain Rh system antibodies.

The subdivision of O_h persons into two classes, as described here, may be an oversimplification, and it is anticipated that other stages between total suppression and full expression of H, A, and B either will be found, or have already been described under other names.

In this study, the i, Sd^a, Le^a and Le^x antigens on O_h red blood cells appeared normal. However, it is already known that the Lewis system antigens reside on the same polysaccharide chains as the I, H, A, and B antigens, and i and Sd^a could well be similarly situated. Our studies included only one example each of the antibodies that detect i, Sd^a, and Le^a and only two that define Le^x. It may be that with multiple examples of these antibodies some of the antigens that they detect will be shown to vary on O_h red blood cells.

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Phyllis P. Moores, B.Sc., Scientific Officer, The Natal Blood Transfusion Service, P.O. Box 2356, 149 Prince Street, Durban, South Africa.

Peter D. Issitt, F.I.M.L.T., L. I. Biol., Director of Laboratories and Assistant Professor in Research Surgery, and Beverly G. Pavone, M. T. (ASCP) Research Technologist, The Paul I. Hoxworth Blood Center of the University of Cincinnati, 3231 Burnet Avenue, Cincinnati, Ohio 45229. (Reprints)

Barbara G. McKeever, M.S., M.T. (ASCP) SBB, Technical Director, Northern Ohio Red Cross Blood Center, 1227 Prospect, Cleveland, Ohio 44114.

Study 6

SEROLOGICAL STUDIES OF ANTI-A+B+H ALLO-ANTIBODIES IN THE SERA OF THREE NATAL 'BOMBAY' O_h INDIANS

11.1 INTRODUCTION

Few technical details are available in the literature of studies with anti-A+B+H allo-antibodies in the sera of O_h individuals. However, Parkin (1956),who examined the allo-antibodies in the sera of two Irish O_h siblings and commented subsequently that her findings were remarkably constant, and Dzierzkowa- Borodej, Meinhard, Nestorowicz and Pirog (1972), who investigated them in the sera of two Polish O_h siblings, included tables in their reports in which the results suggested strongly that the siblings did not have simple mixtures of anti-A, anti-B and anti-H antibodies in their sera. Parkin (1956) commented as well that the Lewis phenotypes of the cells used by her to absorb the Irish siblings' sera had not been significant in this context.

In this chapter, the studies made with the allo-antibodies of three Natal O_h Indians, whose immunological backgrounds varied, will be described. The studies included saliva inhibition tests, and absorbtion-elution tests which went further than the single absorbtion and elution studies of Parkin (1956) and of Dzierzkowa-Borodej *et al.* (1972). The results showed that anti-H, rather than anti-A or anti-B, was almost certainly the most serologically significant antibody specificity in the Indians' sera. Moreover, surprisingly, in each instance a stronger anti-H antibody was recovered in the eluate from the A_1 than from the B cells and from the B than from the O cells. This finding contrasted strangely with the known agglutination by anti-H in A_1 individuals, and by *Ulex* anti-H lectin, of A_1, B and O cells, and therefore also the expected absorbtion of these reagents by A_1, B and O cells, from strong to weak in the order $O \rightarrow A_2 \rightarrow B \rightarrow A_1$ (Race and Sanger, p 50, 1975).

11.2 MATERIALS AND METHODS

11.2.1 The Subjects

Samples of serum from a Natal O_h Indian man and two Natal O_h Indian women, all of whom were known not to have received transfusions of blood, were used in this study.

The Indian man, M. Gov. (69), was a healthy, Tamil-speaking adult of about 25 years of age. The O_h phenotype of his cells was recognised following the donation of his first unit of blood at a Natal Blood Transfusion donation clinic in 1967. The ABO group unexpressed on his cells was O and the Lewis phenotype of his cells was Le(a+b–). The anti-A+B+H allo-antibodies in his serum confirmed, by agglutinating A_1, B and O cells to approximately equal titres in parallel titrations, that he belonged to the category of 'typical' O_h individuals. The titres of his antibodies were the lowest recorded in the three Indians.

The first Indian woman, Mrs P.N. (30), spoke Telegu and is described in further detail in Chapter 7. Approximately 55 years of age, she had a carcinoma, was anaemic, and her pregnancies had numbered at least seven. The ABO group unexpressed on her cells was either A_1, B or A_1B and the Lewis phenotype of her cells was Le(a+b–). The approximately equal titres of her anti-A+B+H allo-antibodies with A_1, B and O cells showed that she, too, was a 'typical' O_h individual. In her case, however, the antibody titres were higher than those of M. Gov.

The second Indian woman, Mrs L. Dew., who spoke Hindi, was 17 years of age, healthy and pregnant for the first time. The O_h phenotype of her cells was discovered in 1969 when a sample of her blood was received for routine antenatal tests. A family study failed to reveal the ABO group unexpressed on her cells and the Lewis phenotype of her cells was Le(a+b–). The approximately equal titres with A_1, B and O cells of her anti-A+B+H allo-antibodies confirmed that her cells were also 'typical' O_h. In due course, Mrs L. Dew. was delivered of a group B infant who suffered mildly from haemolytic disease of the newborn. The eluate made from the infant's cord cells contained an antibody at first identified as anti-B but which was later re-identified as anti-H. Anti-H was also identified in the cord serum. The titres of Mrs L. Dew.'s anti-A+B+H allo-antibodies were the highest recorded in the three Indians.

11.2.2 The cells and sera

The A_1, A_2, B and O cells used in this study were from 475 ml donations of blood in ACD from White donors, and titrations confirmed that their cells were agglutinated by *Ulex* anti-H lectin to the titres expected in relation to their ABO groups. The Lewis phenotype of the group O donor's cells was Le(a+b–). The donations were stored at 4°C and the method of preparing the packed cells and saline and

0,5% bromelin-treated cells from them are described in Chapter 2. The O_h cells, donated by another Natal O_h Indian, were stored at −28°C in glycerol freezing solution (Mollison, p 726, 1979) and were dialysed in saline (Weiner, 1961) for use as required. The samples of serum used from the three Natal O_h Indians were not of the same age but had all been stored continuously at −28°C and appeared in excellent condition. The sera were not inactivated before use as complement was no longer demonstrable in them.

11.2.3 The tests

The saliva inhibition titration technique has been described in Chapter 2. The salivas were from secretors of A and H, B and H, and H substances, and an equal volume of the appropriate saliva was added to all the dilutions of serum in each titration instead of to the neat serum only.

The absorbtion-elution tests were conducted as follows. Three volumes of serum were prepared for use from each O_h Indian. The first volume was absorbed with an equal volume of packed A_1 cells four times, twice at 4°C, once at 37°C and once at 22°C, for periods of one hour each. The second volume was absorbed in the same way with packed B cells and the third volume with packed O cells. The absorbed sera were recovered and stored at −28°C until required, and the packed cells from the second, third and fourth absorbtions were discarded.

The packed cells from the first absorbtions were washed six times with saline at 4°C and in each instance part of the sixth saline wash was retained. The washed cells were eluted into saline by the 56°C heat technique of Landsteiner and Miller (1925), this elution method having been chosen specifically in order that the antibodies in the eluates might be directly compared with those recovered, using the same method, by Parkin (1956) and Dzierzkowa-Borodej *et al.* (1972). The elution technique of Landsteiner and Miller (1925) is still used extensively, and it is quoted as a standard method in the following textbooks: Applied Blood Group Serology (P.D. Issitt and C.H. Issitt, 1975, p 28), Technical Manual of the American Association of Blood Banks (Publ. J.B. Lippincott Co. Philadelphia, USA, p 186) and Blood Transfusion in Clinical Medicine (P.L. Mollison, 1979, p 478). The elution procedure provided nine eluates, or the eluate I samples, each of which was now subdivided into four portions.

The first portions of the eluate I samples were all absorbed once with an equal volume of packed A_1 cells, the second portions absorbed once with packed B cells

and the third portions once with packed O cells, all at 4°C for one hour. The cells were washed six times with saline at 4°C and, again, part of the sixth saline wash was retained. The cells were eluted into saline by the 56°C technique of Landsteiner and Miller (1925), providing 27 further eluates, or the eluate 2 samples.

The nine unabsorbed fourth portions of eluates 1.1 to 1.9, the 27 eluate 2 samples and the 36 saline wash samples were tested with saline suspensions of A_1, B and O cells by the indirect antiglobulin technique, using a broad-spectrum antiglobulin reagent as described in Chapter 2. The results were read both macro- and microscopically, and none of the 36 sixth saline wash samples were found to have agglutinated these cells.

Eluates 1.3, 1.6 and 1.9 were tested as well with 0,5% bromelin-treated A_1, B and O cells, and the nine absorbed serum samples were tested by the indirect antiglobulin technique with saline suspensions of these cells.

11.3 RESULTS

11.3.1 The saliva inhibition titrations

Table 11.1 shows the findings in this study. The reciprocal of the highest dilutions in the titrations to which saline instead of saliva had been added and in which agglutinated cells could still be detected represented the titres of the unabsorbed anti-A+B+H antibodies in the sera of the three Indians. The titres were one dilution tube lower than those which would have been expected had saline not been added to these titrations. The titres of the antibodies of M. Gov. were seen to be 64 with A_1 cells, 32 with B cells and 64 with O cells; those of Mrs P.N. to be 128 with A_1 cells, 256 with B cells and 256 with O cells, and those of Mrs L. Dew. to be 256 with A_1 cells, 512 with B cells and 512 with O cells.

The titres detected after A_1 cells and saliva containing B and H substances, and after B cells and saliva containing A and H substances, had been added to the titrations of the O_h Indians' sera represented the titres of their specific anti-A and anti-B antibodies. This was due to their anti-H antibodies having been inhibited in these titrations by H substance. The titre of M. Gov.'s anti-A was seen to be 32 and of his anti-B 16, the titres of Mrs P.N.'s anti-A and anti-B were both seen to be 64, and the titre of Mrs L. Dew.'s anti-A was seen to be 128 and of her anti-B 32. As their anti-A and anti-B titres were all lower than their anti-H titres (64, 256 and 512 respectively, observed in the control titrations with O cells), their anti-A and anti-B were consequently not seen to be their most serologically significant antibodies. Moreover, the higher titres obtained in the control titrations with the B and O, rather than with the

TABLE 11.1

Saliva inhibition tests with anti-A+B+H antibodies
in the sera of three Natal O_h Indians

Sera	Cells	Substrate	Anti-A+B+H antibodies Dilutions 1	2	4	8	16	32	64	128	256	512	Score	Apparent antibody specificity
	A_1	A+H	–	–	–	–	–	–	–	–	–	–	0	
	A_1	B+H	4	4	4	2	1	(2)	–	–	–	–	44	Anti-A
	A_1	Saline	4	4	4	3	2	1	(2)	–	–	–	54	Anti-A+H
	B	B+H	–	–	–	–	–	–	–	–	–	–	0	
M. Gov. (69)	B	A+H	4	3	2	1	(2)	–	–	–	–	–	34	Anti-B
	B	Saline	4	4	3	3	1	(2)	–	–	–	–	46	Anti-B+H
	O	A+H	(3)	–	–	–	–	–	–	–	–	–	2	
	O	B+H	–	–	–	–	–	–	–	–	–	–	0	
	O	H	(2)	–	–	–	–	–	–	–	–	–	1	
	O	Saline	4	4	4	3	2	1	(2)	–	–	–	54	Anti-H
	A_1	A+H	–	–	–	–	–	–	–	–	–	–	0	
	A_1	B+H	4	4	4	3	1	(3)	(1)	–	–	–	47	Anti-A
	A_1	Saline	4	4	4	3	3	2	1	(2)	–	–	64	Anti-A+H
	B	B+H	–	–	–	–	–	–	–	–	–	–	0	
Mrs P.N. (30)	B	A+H	4	4	3	3	2	1	(1)	–	–	–	53	Anti-B
	B	Saline	4	4	4	4	4	4	3	2	(3)	–	80	Anti-B+H
	O	A+H	(2)	–	–	–	–	–	–	–	–	–	1	
	O	B+H	–	–	–	–	–	–	–	–	–	–	0	
	O	H	(2)	–	–	–	–	–	–	–	–	–	1	
	O	Saline	4	4	4	4	4	4	3	2	(3)	–	80	Anti-H
	A_1	A+H	(2)	(1)	–	–	–	–	–	–	–	–	1	
	A_1	B+H	4	4	4	4	3	2	1	(1)	–	–	63	Anti-A
	A_1	Saline	4	4	4	4	4	3	2	1	(2)	–	74	Anti-A+H
	B	B+H	–	–	–	–	–	–	–	–	–	–	0	
Mrs L. Dew.	B	A+H	4	4	3	2	(3)	(1)	–	–	–	–	40	Anti-B
	B	Saline	4	4	4	4	4	4	3	2	(3)	(1)	80	Anti-B+H
	O	A+H	3	2	1	(1)	–	–	–	–	–	–	23	
	O	B+H	–	–	–	–	–	–	–	–	–	–	0	
	O	H	1	(3)	(2)	–	–	–	–	–	–	–	8	
	O	Saline	4	4	4	4	4	4	3	2	(3)	(1)	80	Anti-H

A_1 cells, using the serum of Mrs P.N. and Mrs L. Dew., supported the view, in these two Indians at least, that the identity of their serologically most significant antibody was anti-H. The anti-H in Mrs L. Dew.'s serum, which was not inhibited as completely as the anti-H in the sera of the other two Indians by the salivas containing A+H and H substances, showed by this as well that it was almost certainly her most serologically potent antibody. The complete inhibition of anti-H in the sera of all three Indians, noted with the saliva containing B+H substances, was attributed to the donor being a natural secretor of a greater quantity of saliva substances than the other two donors.

11.3.2 The absorbtion-elution studies

Table 11.2 shows that the antibodies in eluates 1.1 and 1.7 agglutinated A_1, B and O cells almost all equally strongly and that the antibodies in all the other eluate 1 samples agglutinated them as if they had anti-B+H or anti-H specificity. The anti-H specificity of these antibodies was identified by their agglutination of A_1, B and O cells from strong to weak in the order of O → B → A_1 and their non-agglutination of the O_h control cells.

After absorbtion with A_1 cells, eluates 1.1.d, 1.4.d, and 1.7.d and, after absorbtion with O cells eluates 1.1.f, 1.4.f, 1.5.f and 1.7.f, all appeared to contain anti-B. The curious elution of anti-B from A_1 cells may have been due to the Matuhasi-Ogata phenomenon (Ogata and Matuhasi, 1962 and 1964) but Bird (1953 and 1954) noticed that anti-A,B antibodies which agglutinated B more strongly than A cells were eluted readily from A cells, while those which agglutinated A more strongly than B cells were not eluted readily from B cells. Since Bird's observations with anti-A,B were remarkably similar to mine with the anti-A+B+H antibodies of the O_h Indians, in my tests the only evidence that anti-A had been weakly eluted from B cells had been seen in eluates 1.2.e and 1.2.f, and anti-A and anti-B had not been eluted equally readily from B and A cells respectively, the likelihood that the Matuhasi-Ogata phenomenon was the correct solution was discarded.

Eluates 1.1.e, 1.2.e and 1.4.e absorbed with B cells, and eluates 1.1.f, 1.2.f and 1.4.f absorbed with O cells, were all seen to contain anti-A. However, the weak agglutination of A_1 cells by eluates 1.4.d, 1.7.d, 1.8.d and 1.9.d absorbed with A_1 cells and by eluates 1.8.f and 1.9.f absorbed with O cells was considered rather to be a manifestation of anti-H, as they had agglutinated O more strongly than B and B more

TABLE 11.2

Results of tests with eluate 1 samples obtained from A_1, B and O cells used to absorb the sera of three Natal O_h Indians, with the same eluates after they had been absorbed with A_1, B and O cells and with the eluate 2 samples obtained subsequently from these cells

Sera	Cells used to absorb sera	Eluate 1 samples	Results Eluate 1 samples				Cells used to absorb eluate 1 samples	Absorbed eluate 1 samples	Results Absorbed eluate 1 samples			Eluate 2 samples	Results Eluate 2 samples		
			A_1	B	O	O_h			A_1	B	O		A_1	B	O
							A_1	d	–	1	–	1	–	–	–
	A_1	1	4	3	4	–	B	e	2	–	–	2	–	–	–
							O	f	2	1	–	3	–	–	–
							A_1	d	–	–	–	4	–	–	<1
M. Gov. (69)	B	2	1	1	2	–	B	e	<1	–	–	5	–	1	1
							O	f	<1	–	–	6	–	–	<1
							A_1	d	–	–	<1	7	–	–	–
	O	3	1	1	2	–	B	e	–	–	–	8	–	–	1
							O	f	–	–	–	9	–	–	–
							A_1	d	<1	3	2	10	<1	2	4
	A_1	4	2	4	4	–	B	e	<1	–	<1	11	–	1	3
							O	f	<1	4	–	12	–	<1	1
							A_1	d	–	2	2	13	–	1	3
Mrs P.N. (30)	B	5	1	4	4	–	B	e	–	<1	<1	14	–	1	2
							O	f	–	1	<1	15	–	<1	1
							A_1	d	–	1	2	16	–	1	1
	O	6	<1	4	4	–	B	e	–	–	<1	17	–	<1	1
							O	f	–	–	<1	18	–	–	–
							A_1	d	<1	4	2	19	<1	3	4
	A_1	7	4	4	4	–	B	e	–	1	1	20	–	1	3
							O	f	–	2	–	21	–	1	1
							A_1	d	1	4	4	22	<1	4	3
Mrs L. Dew.	B	8	1	4	4	–	B	e	–	1	1	23	<1	2	3
							O	f	<1	1	1	24	<1	2	3
							A_1	d	1	4	4	25	1	3	3
	O	9	1	4	4	–	B	e	–	2	2	26	<1	2	3
							O	f	<1	2	2	27	<1	2	3

strongly than A_1 cells. A stronger anti-H was apparent in the absorbed eluates 1.4.d, 1.5.d, 1.6.d, 1.7.d, 1.8.d and 1.9.d than in the absorbed eluates 1.4.e, 1.5.e, 1.5.f, 1.6.e, 1.6.f, 1.7.e, 1.8.e, 1.8.f, 1.9.e and 1.9.f, and this was believed to show that the A_1 cells used to absorb them had removed less anti-H than the B cells and the B cells less anti-H than the O cells.

All the eluate 2 samples agglutinated O cells more strongly than B and B more strongly than A_1 cells. Consequently they were all believed to contain anti-H. However, except in eluates 2.1 to 2.9, a stronger anti-H had been eluted from the A_1 cells (eluates 2.10, 2.13, 2.16, 2.19, 2.22, 2.25) than from the B cells (eluates 2.11, 2.14, 2.17, 2.20, 2.23, 2.26) and a stronger anti-H from the B than from the O cells (eluates 2.12, 2.15, 2.21). Eluates 2.23, 2.24, 2.26 and 2.27 all appeared to contain anti-H antibodies of almost equal strength; and the agglutination of A_1, B and O cells by eluates 2.1 to 2.9, while not exhibiting exactly the same pattern as anti-H, at least showed that the pattern exhibited had a certain degree of regularity.

Since Mrs L. Dew.'s eluates 2.22 to 2.27 were found to contain stronger anti-H antibodies than her eluates 2.19 to 2.21, while Mrs P.N.'s eluates 2.10 to 2.12 were found to contain stronger anti-H antibodies than her eluates 2.13 to 2.15 and her eluates 2.13 to 2.15 stronger anti-H antibodies than her eluates 2.16 to 2.18, more potent anti-H antibodies appeared to have been recovered in the eluate 1 samples from the B and O than from the A_1 cells used to absorb Mrs L. Dew.'s serum. As a result, in her case, these findings were at variance with my view that anti-H had been recovered from the A_1, B and O cells from strong to weak in the order $A_1 \rightarrow B \rightarrow O$. Although Mrs L. Dew.'s eluate 2 samples were not subjected to further absorbtion-elution tests, the anti-H agglutination pattern seen with her absorbed eluate 1 samples suggested that a similar pattern might have been observed after her eluate 2 samples had been absorbed and tested with A_1, B and O cells. If so, then the pattern, when her eluate 3 samples were tested, was expected to have been the same as the pattern observed when the eluate 2 samples of Mrs P.N. were tested with A_1, B and O cells. This prediction was supported by the evidence that Mrs L. Dew. had a more potent anti-H antibody than Mrs P.N. (see Table 11.1) and by the knowledge that at birth her infant had been affected by haemolytic disease of the newborn due to anti-H.

Table 11.3 shows, by bromelin-treated A_1, A_2, B and O cells having been agglutinated in the order $O \rightarrow A_2 \rightarrow B \rightarrow A_1$, that the antibodies in eluates 1.3 of M. Gov., 1.6 of Mrs P.N. and 1.9 of Mrs L. Dew., almost certainly all contained anti-H.

TABLE 11.3

Results of tests with eluates 1.3, 1.6 and 1.9 recovered from O cells used to absorb the sera of three Natal O_h Indians

Sera	Eluate 1 samples	Bromelin-treated cells			
		A_1	A_2	B	O
Mr M. Gov. (69)	3	–	1	< 1	2
Mrs P.N. (30)	6	–	2	< 1	2
Mrs L. Dew.	9	–	2	1	2

The results obtained when the Indians' sera, absorbed four times with A_1, B or O cells, were tested with A_1, B and O cells are presented in Table 11.4. Separate anti-A and anti-B antibody specificities were evident in all three absorbed sera; but in the absorbed sera from Mrs L. Dew. the absorbtions were seen not to have been complete. In separate tests, the weak antibodies in her absorbed sera were all shown to have anti-H specificity. The finding was confirmed by the stronger agglutination of O than of B and of B than of A_1 cells by her absorbed sera shown in Table 11.4.

11.4 DISCUSSION

As many serologists have described in studies of their own with the anti-A+B+H antibodies in the sera of other O_h people, the results in this chapter showed that the sera of the three Natal O_h Indians all contained separable anti-A, anti-B and anti-H antibodies. In the eluates recovered from the A_1, the B and the O cells used to absorb their sera, the expected anti-A, anti-B and anti-H antibodies were also detected, but in most instances their specificities were not demonstrated clearly until after the eluates had been partially absorbed. However, the antibodies in the second set of eluates made from the cells used to absorb the first set showed, by agglutinating A_1, B and O cells from strong to weak in the order $O \rightarrow B \rightarrow A_1$, that they all had only anti-H specificity.

TABLE 11.4

Results of tests with the sera of three Natal O_h Indians after absorbtion four times with A_1, B or O cells

Sera	Cells used to absorb sera	Results cells A_1	B	O
M. Gov. (69)	A_1	–	4	–
	B	4	–	–
	O	4	4	–
Mrs P.N. (30)	A_1	–	4	–
	B	4	–	–
	O	4	4	–
Mrs L. Dew.	A_1	(1)	4	2*
	B	4	(2)	(3)
	O	4	4	(2)

Key: * Further tests confirmed that the antibody causing less than 4+ agglutination was anti-H

The recovery of anti-H in all the eluate 2 samples in itself was not extraordinary, as A_1, B and O cells were all known to have some H antigen, O more than B cells and B more than A_1 cells (Race and Sanger, p 35, 1975). The most interesting finding in this study, and one which has apparently not been recorded before, was that stronger anti-H antibodies had been recovered in the eluate 2 samples of Mrs L. Dew. and Mrs P.N. from the A_1 than from the B and from the B than from the O cells used to absorb their eluate 1 samples. The anti-H agglutination pattern, which was not as obvious with the antibodies in Mrs L. Dew.'s eluate 2 samples as it had been with those in the eluate 2 samples of Mrs P.N., was attributed to Mrs L. Dew. having a more potent anti-H antibody. The potency of Mrs L. Dew.'s anti-H antibody was observed in the saliva inhibition titrations and tests with her absorbed sera, and her infant had also suffered from haemolytic disease of the newborn due to anti-H.

Possibly her infant, still unborn when her sample of serum was acquired, had been the source of antibody stimulation in her case. The reason anti-H was present in all the eluate 2 samples was thought to be, not that the A_1 had absorbed more anti-H than the B and the B more than the O cells but on being eluted, that the A_1 had yielded up more anti-H than the B and the B more anti-H than the O cells. This may have been due either to the anti-H antibodies having bound more firmly to the O than to the B, and more firmly to the B than to the A_1 cells, or to the smaller proportion of H antigen sites known to exist on the A_1 having been more easily disrupted by the 56°C heat elution procedure than on the B and on the B than on the O cells. The finding seemed to echo Bird's observations (1953 and 1954), and it was also similar to the finding (Voak and Lodge, 1968) that A_2 cord cells absorbed more *Ulex* anti-H lectin and eluted less than A_1 cord cells. In addition, Voak and Lodge (1968) described their results as not being as marked as those of Fischer and Hahn (1935) who had used anti-A and A cells. Finally, it was reminiscent of the familiar elution from cells with some weakly expressed A and B antigens of stronger anti-A and anti-B antibodies, respectively, than from cells with normally expressed A and B antigens (Celano, Levine and Lange, 1957).

The stronger anti-H antibodies eluted from the B than from either the A_1 or the O cells in the eluate 2 samples used to absorb the eluate 1 samples of M. Gov. (Table 11.2) suggested that, in his case, the A_1 cells might not have been able to retain sufficient antibody molecules for enough to be eluted and cause even the O cells to be agglutinated in the tests made later. However, the B cells had apparently been able to do so, and the O cells may have retained them so firmly that they were either not eluted or were eluted from them much less readily.

In the eluate 1 samples of M. Gov., Mrs P.N. and Mrs L. Dew., and in the single set of eluates recovered by Dzierzkowa-Borodej *et al.* (1972) from A_1, B and O cells used to absorb the sera of her two Polish O_h siblings, the anti-H specificity of the antibodies was thought to have been masked by their own strength and by the anti-anti-A and anti-B antibodies also present in them. However, in the single set of eluates recovered by Parkin (1956), from the A_1, B and O cells used by her to absorb the sera of her two Irish O_h siblings, the anti-H specificity of the antibodies was demonstrable, in spite of the presence of anti-A, eluted as well from the A_1 cells. Parkin (1956) must either have failed to observe the anti-H specificity of the antibodies in her eluates or have deemed the antibodies to be too weak for comment.

The results in this study revealed that there were marked differences between the anti-A+B+H antibodies in the sera of M. Gov., Mrs P.N. and Mrs L. Dew. In M. Gov.'s serum, perhaps because he was a male, the antibody titres recorded were the lowest, and his anti-H antibodies appeared the least concentrated. In Mrs P.N.'s serum, the antibody titres recorded were higher, and her anti-H antibodies appeared relatively more concentrated, as befitted an elderly woman who had been pregnant with incompatible fetuses several times in the past. In Mrs L. Dew.'s serum, the antibody titres recorded were the highest, and her anti-H antibodies appeared exceptionally concentrated, as befitted a currently pregnant woman whose infant later suffered from haemolytic disease of the newborn due to anti-H. The observation that A_1 cells absorbed less but yielded up more anti-H than B cells and B cells absorbed less but yielded up more anti-H than O cells also suggested that group O infants might be at an equal, if not at a greater, risk than their group A, B and AB siblings from haemolytic disease of the newborn due to their mother's anti-A+B+H antibodies.

Perhaps it should be mentioned here that this entire study was repeated in 1980 for confirmation and that the same results were obtained. The double (and perhaps even treble) elution procedure described was also seen unexpectedly to have provided a method of obtaining anti-H in pure form from anti-A+B+H antibodies.

It might be interesting, on some future occasion, to study in a similar manner the antibodies obtained in the eluates recovered by a different elution method, such as the ether technique of Vos and Kelsall (1956).

11.5 SUMMARY

The most serologically significant of the anti-A+B+H antibodies in the sera of three Natal O_h Indians was found to be their anti-H antibody. This was revealed when anti-H was detected in almost all the eluates recovered from the A_1, B and O cells used to absorb the initial set of eluates recovered from A_1, B and O cells used to absorb their sera. The anti-H antibodies in these eluates, which agglutinated A_1, B and O cells from strong to weak in the order $O \rightarrow B \rightarrow A_1$, were surprisingly found to be eluted from strong to weak in the order $A_1 \rightarrow B \rightarrow O$. A possible explanation for this so far apparently unrecorded finding was advanced.

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Study 7

PARA-BOMBAY PHENOTYPES IN THE NATAL INDIANS

12.1 INTRODUCTION

Para-Bombay red cells are not agglutinated by anti-H and they are either weakly or are not agglutinated by anti-A, anti-B and anti-A,B reagents. Their A, B or AB antigens are also sometimes only detected by means of absorption-elution and other studies. The secretors secrete normal amounts of A, B and H substances in their saliva, and their serum often contains an anti-'O'-like antibody, not inhibited by secretor saliva, which may be anti-HI. The non-secretors usually have anti-H in their serum (Race and Sanger, p 26, 1975; Watkins, 1980).

Not everyone uses the same terminology for para-Bombay cells. The term A_h was chosen for the first example by Levine, Uhlíř and White, who discovered it in 1961; and Beranová, Prodanov, Hrubiško and Smálik reported the first cells with a B_h phenotype in 1969. Solomon, Waggoner and Leyshon (1965), however, and Kitahama, Yamaguchi, Okubo and Hazama (1967), used the terms A_m^h, B_m^h and O_m^h for their apparently weaker examples. In 1970, Hrubiško, Laluha, Mergancová and Zakovicová suggested using the terms A_{Hm} and B_{Hm} for the para-Bombay cells which were agglutinated by anti-A, anti-B and anti-A,B reagents, and using the terms O_{Hm}^A, O_{Hm}^B, O_{Hm}^{AB} and O_{Hm} when the cell antigens had to be identified indirectly. Later, Race and Sanger (p 26, 1975) suggested retaining A_h and B_h for the cells of the non-secretors and using A_m^h and O_{Hm}^A etc. for the secretors. The suggestions made by Race and Sanger have been followed in this thesis.

Since the secretors clearly have *H* genes, in these individuals, the para-Bombay red cell phenotype is regarded as being due to the production of less than the normal amount of H antigen on the membranes of their cells. For some reason, production of their *H* gene-specified α-2-L-fucosyltransferase is partially inhibited and, as the *A* and *B* gene-specified glycosyltransferases are present in normal amounts in their sera and haemopoetic tissues, all of the H antigen-specific glycoprotein and glycosphingolipid of their cells is apparently converted to A, B or AB antigens (Watkins, 1980). The cause, in the para-Bombay secretors, has been attributed to the inheritance of a gene (or genes) which may be a mutant regulator. Solomon, Waggoner and Leyshon

(1965) suggested that this gene and its allele should be called *Z* and *z* respectively, and para-Bombay people may therefore have *zz* genes. Mulet, Cartron, Lopez and Salmon (1978) suggested as well that a more appropriate notation for para-Bombay secretors would be O^{A}_{HZ}, O^{B}_{HZ} etc.

In this chapter, the results of my studies with three Natal Indians who had para-Bombay cells are presented. Their phenotypes were identified as O^{A}_{Hm}, O^{B}_{Hm} and O_{Hm} (or perhaps O^{A}_{HZ}, O^{B}_{HZ} and O_{HZ}) respectively.

12.2 CASE HISTORIES

12.2.1 Mrs G.N. (O^{A}_{Hm}) was discovered in 1977 when she donated a unit of her blood at an NBTS blood donation clinic in Durban. An antibody which agglutinated all of a collection of eight panel cells of known groups and two group O cord cells, but not her own cells, was detected in her serum. The antibody was identified as anti-H, but her cells appeared to be group O. Her blood samples were therefore referred to me for further studies. Mrs G.N. was in excellent health, and her home language was Tamil.

12.2.2 Miss S.M. (O^{B}_{Hm}), was the 18 months-old infant daughter of Moslem parents. She was found in 1977 as well, while in hospital for corrective hip surgery. Although she was apparently group O, no units of O blood crossmatched for her had so far been found compatible with the saline- and enzyme-reacting antibody in her serum.

12.2.3 Mrs G.G. (O_{Hm}) was encountered first in 1967 when she was 26 weeks pregnant. She was 36 years of age and was in hospital suffering from nephritis and severe anaemia. Her cells were apparently group O but 54 units of O blood has been crossmatched for her and all had been found incompatible. Further blood samples were received from her in 1968, four weeks after her infant had been delivered, and also when she was again pregnant, in 1969. However, her phenotype was not identified correctly until 1977.

As far as it was known, Mrs G.N., Miss S.M. and Mrs G.G. were not related.

12.3 MATERIALS AND METHODS

The blood samples from Mrs G.N., from Miss S.M. and members of her family and

from Mrs G.G. and her family were of clotted blood and were prepared for testing as indicated in Chapter 2. The saliva samples obtained from Miss S.M. and her family members and from Mrs G.G. were processed correctly by the technologist in charge at the blood bank in their respective hospitals before being despatched to my laboratory. The reagents used, which were all of commercial origin or had been obtained and standardised by myself according to customary strict criteria, were also used by the techniques described in Chapter 2. The anti-Le^b reagents used had been tested with the saliva of a secretor of H substance only and had been found to be type anti-anti-Le^{b1}.

12.4 RESULTS

12.4.1 *Mrs G.N.* The red cells of Mrs G.N. were not agglutinated by standard anti-A or anti-B reagents in saline or one-stage enzyme tests at 20°C, but were agglutinated weakly by anti-A,B reagents and by the anti-A+B+H antibodies of several 'Bombay' O_h donors in saline and one-stage enzyme tests at this temperature (Table 12.1, parts a and b). Her cells were also agglutinated weakly by some but not all the anti-A reagents and not by the anti-B reagents used, in saline tests at 10°C, and they were agglutinated by all the anti-A and by none of the anti-B reagents used, in saline tests at 4°C. A known high-titre immune anti-A, but not a known high-titre immune anti-anti-B, reagent agglutinated her cells weakly in one-stage enzyme tests at 20°C (Table 12.1, part a), and her cells were not agglutinated by *Ulex* anti-H lectin (Table 12.1, part c).

After exposing Mrs G.N.'s red cells to anti-A and anti-B reagents at 20°C for two hours, anti-A, and not anti-B, was readily recovered from them in eluates made in 6% bovine albumin by the 56°C technique of Landsteiner and Miller (1925). The I-antigen strength of her cells was not seen to be increased in titrations with three different anti-I reagents, and her serum was found to contain anti-B and IgM cold antibody that agglutinated O and A_2 adult cells moderately well (2+), A_1 adult and O cord cells weakly and failed to agglutinate O_h cells in saline and one-stage enzyme tests (Table 12.1, part d). The auto-antibody control test with her serum was negative. The specificity of the cold antibody in her serum was surprisingly confirmed as anti-H, when it was seen to be inhibited by an equal volume of commercial ABH blood group specific substances and not by an equal volume of saline in parallel tests.

TABLE 12.1

Reactions of red cells and serum of Mrs G.N. with various reagents

Part a

Reagent	Technique	°C	Mrs G.N.	Cells Positive	Negative Control
Anti-A			–	4	–
Anti-B		20	–	4	–
Anti-A,B			(3)	4	–
AB serum			–		
Anti-A	Saline		–	4	–
Anti-A	Saline	10	(2)	4	–
Anti-B	Saline		–	4	–
Anti-A			(3)	4	–
Anti-A		4	(3)	4	–
Anti-B			–	4	–
Anti-A	one stage 0,5% bromelin		–	4	–
Anti-B	one stage 0,5% bromelin	20	–	4	–
Anti-A,B	one stage 0,5% bromelin		1	4	–
AB serum	one stage 0,5% bromelin		–		–
Anti-A	one stage 0,25% ficin		–	4	–
Anti-B	one stage 0,25% ficin	20	–	4	–
Anti-A,B	one stage 0,25% ficin		1	4	–
AB serum	one stage 0,25% ficin		–		
Immune anti-A	0,5% Bromelin	20	2	4	–
Immune anti-B	0,5% Bromelin		–	4	–

Part b

'Bombay' anti-A+B+H sera	°C	Mrs G.N. Saline	Mrs G.N. 0,5% brom.	O cells Saline	O cells 0,5% brom.	O_h cells Saline	O_h cells 0,5% brom.
Mrs P. Gov. 165		(3)	2	4	4	–	–
S. Red. 215		(1)	1	4	4	–	–
R. Red. 223	20	(3)	1	4	4	–	–
Mr S. Nai. 212		–	(3)	3	4	–	–
Mrs P.C. 121		–	(3)	4	4	–	–

Part c

	Ulex anti-H dilutions 1	2	4	6	16	32	64
Mrs G.N.	–	–	–	–	–	–	–
A_1	1	(3)	(3)	(1)	–	–	–
A_2	4	4	4	3	3	1	(2)
B	2	2	1	1	(1)	–	–
O	4	4	4	3	1	(2)	(2)
A_1B	(3)	(±)	–	–	–	–	–
O_h	–	–	–	–	–	–	–

Part d

Cells	Mrs G.N. Serum Saline 20°C	0,5% brom. 20°C	0,25% ficin 20°C
A_1	(2)	(2)	1
A_2	2	2	3
B	4	4	4
O	2	2	4
O_h	–	–	–
Cord O	1	1	2

Other groups on Mrs G.N.'s cells were identified as follows:

MNSs, P_1, CDe/cde (Rh_1rh), hr^S+, hr^B+, C^W-, Lu(a–), K–, Kp(a–), Le(a–b+), Fy(a+), Jk(b+), I+, Sd(a+).

A saliva sample was not received from Mrs G.N. and, unfortunately, her family was not available for a family study. However, since the Lewis phenotype of her cells was Le(a–b+), she was presumed to be a secretor of ABH substances.

12.4.2 *Miss S.M.* Like the cells of Mrs G.N., the cells of Miss S.M. were not agglutinated by standard anti-A or anti-B reagents in saline or one-stage enzyme tests at 20°C, but they were agglutinated weakly by anti-A,B reagents (Table 12.2, part a). These tests were not repeated at lower temperatures in her case. Her cells were also not agglutinated by *Ulex* anti-H lectin in saline or in one-stage 0,5% bromelin tests at 20°C (Table 12.3, part a), but her cells were agglutinated strongly (4+) in saline and in one-stage 0,5% bromelin tests by some of the examples of anti-A+B+H, were not agglutinated in saline but were agglutinated weakly in one-stage 0,5% bromelin tests by some of the examples, and were not agglutinated by either technique by other examples of this antibody (Table 12.3, part b). However, the anti-A+B+H antibodies of R. Red. (216), which had reacted 4+ with her cells in saline tests, agglutinated them only to titre 4, while those of Mrs P. Gov. (165), which had not reacted with her cells, agglutinated the B and O control cells to titre 128 (Table 12.3, part a).

After the cells of Miss S.M. had been exposed to anti-A and anti-B for two hours at 20°C, anti-B but not anti-A was recovered readily from them in eluates made in saline by the 56°C heat technique of Landsteiner and Miller (1925). The I antigen strength of her cells was not found to be increased. Her serum was seen to contain a strong anti-A allo-antibody and a weak IgM cold antibody that agglutinated all the group O cells with which it was tested 1+ in saline tests and 3+ both in one-stage 0,5% bromelin and in one-stage 0,25% ficin tests at 20°C. Group B adult and group O cord cells were agglutinated by this antibody weakly, and the serum auto-antibody control test was negative (Table 12.2, part b). The identity of her cold antibody, the titre of which was 8 with O cells in one-stage 0,25% ficin tests, was thought to be anti-H; but no further studies were possible as the small quantity of her serum available had to be retained for crossmatching tests. Saliva inhibition tests showed that Miss S.M. was a secretor of B and H but not of A substances.

TABLE 12.2

Reactions of red cells and serum of Miss S.M. with various reagents

Part a

Reagent	Technique	°C	Miss S.M.	Cells	
				Positive control	Negative control
Anti-A			–	4	–
Anti-B	saline	20	–	4	–
Anti-A,B			(1)	4	–
Anti-A	one-stage		–	4	–
Anti-B	0,5%	20	–	4	–
Anti-A,B	bromelin		1	4	–
Anti-A	one-stage		–	4	–
Anti-B	0,25%	20	–	4	–
Anti-A,B	ficin		1	4	–

Part b

Cells	Miss S.M. serum	
	Saline 20°C	0,5% brom. 20°C
A_1	4	4
A_2	3	4
B	(2)	1
O	1	3
O_h	–	–

TABLE 12.3

Reactions of anti-A+B+H with cells of Miss S.M., group O and group B adult cells and group O cord cells

Part b

'Bombay' anti-A+B+H	Miss S.M. cells		Adult O cells		Cord O cells		O_h cells	
	Saline	0,5% brom.	Saline	0,5% brom.	Saline	0,5% brom.	Saline	0,5% brom.
Mrs P. Gov. 165	–	–	4	4	4	4	–	–
,, 230	–	1	4	4	4	4	–	–
Mrs B. Gov. 166	1	2	4	4	4	4	–	–
,, 188	1	2	4	4	4	4	–	–
,, 231	2	4	4	4	4	4	–	–
S. Red. 203	–	3	4	4	4	4	–	–
,, 215	–	4	4	4	4	4	–	–
,, 227	(1)	4	4	4	4	4	–	–
R. Red. 204	2	4	4	4	4	4	–	–
,, 216	4	4	4	4	4	4	–	–
,, 223	4	4	4	4	4	4	–	–
Mrs L. Dew. 195	3	4	4	4	4	4	–	–
,, 219	3	4	4	4	4	4	–	–
Miss A. Gov. 214	–	(2)	4	4	4	4	–	–
M. Gov. 153	–	1	4	4	4	4	–	–
M. Gov. 213	(1)	1	4	4	4	4	–	–
S. Nai. 169	–	–	4	4	4	4	–	–
S. Nai. 212	–	–	4	4	4	4	–	–
S. Nai. 168	–	–	4	4	4	4	–	–
S. Nai. 211	–	(1)	4	4	4	4	–	–
A. Nar. 202	3	3	4	4	4	4	–	–
Mrs P.C. 121	1	3	4	4	4	4	–	–

Part a

Reagent	Technique	Cells	Dilutions 1	2	4	8	16	32	64	128	256	512
		Miss S.M.	–	–	–	–	–	–	–	–	–	–
	Saline	A_1	1	(3)	(2)	(1)	–	–	–	–	–	–
Ulex	20°C	A_2	4	4	4	4	3	2	1	(2)	–	–
anti-H		B	1	1	(3)	(2)	–	–	–	–	–	–
		O	4	4	4	4	3	2	1	(2)	–	–
Ulex	one-stage	Miss S.M.	–	–	–	–	–	–	–	–	–	–
anti-H	0,5%	O	4	4	4	4	4	4	4	4	2	1
	brom. 20°C	O_h	–	–	–	–	–	–	–	–	–	–
R. Red		Miss S.M.	4	2	1	–	–	–	–	–	–	–
216	Saline	B	4	4	4	3	3	2	1	(2)	–	–
anti-A+B+H	20°C	O	4	4	4	4	3	2	1	(2)	–	–
		O_h	–	–	–	–	–	–	–	–	–	–
Mrs P. Gov.		Miss S.M.	–	–	–	–	–	–	–	–	–	–
165	Saline	B	4	4	4	4	4	2	1	1	(2)	–
anti-A+B+H	20°C	O	4	4	4	4	2	1	1	(2)	–	–
		O_h	–	–	–	–	–	–	–	–	–	–

Other groups on Miss S.M.'s cells were identified as follows:

MN, U+, P_2, CDe/CDe (Rh_1Rh_1), hr^S+, hr^B+, Lu(a–), Le(a–b+).

The samples of blood and saliva from members of Miss S.M.'s family showed, in the appropriate tests, that her father, I-1, was O and secreted H but not A or B substances, and that her mother, I-2, and two elder sisters, II-1 and II-2, were all B and secreted B and H but not A substances (Figure 12.1). As expected, the cells of I-1 were agglutinated strongly, and the cells of I-2 and II-1 weakly, by *Ulex* anti-H lectin, but the cells of II-2 were agglutinated unexpectedly almost as strongly as the control A_2 cells by this reagent (Table 12.4). The Lewis phenotypes of the B as well as the O members in this family were determined with confidence using the anti-Le^a, anti-Le^{bL} and anti-Le^x reagents.

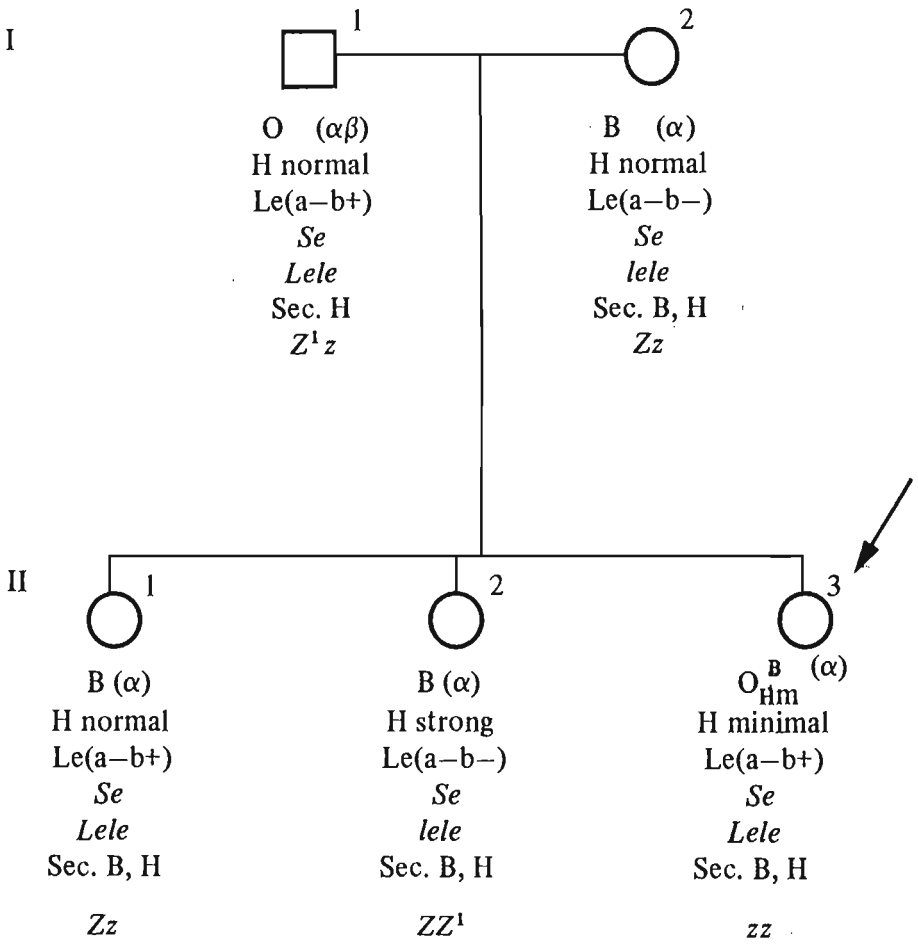
TABLE 12.4

Reactions of cells of members of Miss S.M.'s family with *Ulex* anti-H lectin

Cells	Ulex anti-H dilutions						
	1	2	4	8	16	32	64
I-1	4	4	4	4	4	2	1
I-2	1	(2)	–	–	–	–	–
II-1	1	1	(2)	–	–	–	–
II-2	4	4	3	3	1	(3)	–
A_1	(2)	–	–	–	–	–	–
A_2	4	4	4	4	2	1	–
B	1	(2)	–	–	–	–	–
O	4	4	4	4	3	2	1
A_1B	(1)	–	–	–	–	–	–

Figure 12.1

Family of Miss S.M., phenotype O^{B}_{Hm}



Key: ↙ = Proposita

Z, Z^1, z = Suggested regulator genes

12.4.3 *Mrs G.G.* The cells of Mrs G.G. were not agglutinated by standard anti-A or anti-B or by high titre immune anti-A and anti-B reagents. However, unlike the cells of Mrs G.N. and Miss S.M., her cells were also not agglutinated by anti-A,B reagents. *Ulex* anti-H lectin failed to agglutinate her cells, and her cells were agglutinated weakly by three examples of anti-A+B+H in saline tests at 20°C (Table 12.5, part a). No tests were made at temperatures lower than 20°C, and her cells were not subjected to absorption-elution studies. The I antigen strength of her cells was not found to be increased in titrations with several different anti-I reagents.

Mrs G.G.'s saliva did not inhibit a single dilution (1 in 8 in saline) of anti-A or anti-B, but it did inhibit a single dilution of *Ulex* anti-H lectin (diluted 1 in 4 in saline). In the subsequent titration, in which her saliva was diluted serially in saline and the anti-H reagent was used undiluted, she was seen to secrete as much H substance as the control O secretor (Table 12.5, part b). An anti-Le^{bL}, but not an anti-Le^{a}, reagent was inhibited by her saliva, but only the dilutions from 1 to 32 inhibited this antibody, whereas all the dilutions from 1 to 512 of the saliva of the control O secretor did so readily. The anti-Le^{bL} reagent (PPM 39) was known not to be inhibited by the saliva of a secretor of H but of no Lewis substances.

The titres of Mrs G.G.'s allo-antibodies were found to be 128 with A_2 cells and 64 with B cells, in saline titrations at 20°C. The titres therefore confirmed that her cells did not have A or B antigens. Her serum agglutinated adult O cells to titre 4 in a saline titration, and to titre 8 in a one-stage 0,5% bromelin titration, at 20°C. Cord O cells were agglutinated weakly, and the auto-antibody control test was negative. The reaction of her serum with O cells was not inhibited by the addition of an equal volume of the saliva of different O secretors, and one absorption of her serum with O cells removed all her antibody completely. As a result, the identity of her antibody was seen almost certainly to be anti-'O'-like, and it was similar to the antibodies often found in para-Bombay secretors, now thought to be anti-HI (Race and Sanger, p 26, 1975). The antibody was identified again in Mrs G.G.'s serum in 1968, and also in 1969.

In 1967, Mrs G.G.'s cells were typed with other reagents as follows:

MNs, P_1, CDe/CDe ($Rh_1 Rh_1$), Lu(a–), K–, Le(a–b–), Fy(a+), I+

TABLE 12.5

Reactions of red cells, serum and saliva of Mrs G.G. with various reagents

Part a

Reagent	Technique	°C	Cells: Mrs G.G.	Cells: Pos. control	Cells: Neg. control
anti-A			–	4	–
anti-B			–	4	–
anti-A,B	saline	20	–	4	–
anti-A+B+H			(3)	4	–
Ulex anti-H			–	4	–
immune anti-A			–	4	–
immune-anti-B			–	4	–

Part b

Subject	Reagent	Saliva dilutions: 2	4	8	16	32	64	128	256	512
Mrs G.G.	Ulex	–	–	–	–	–	1	2	3	4
O secretor	anti-H	–	–	–	–	–	2	4	4	4
O non-secretor	undiluted	4	4	4	4	4	4	4	4	4
Mrs G.G.		–	–	–	–	–	1	2	3	3
O secretor	anti-Le^b	–	–	–	–	–	–	–	–	–
O non-secretor	undiluted	1	2	3	3	3	3	3	3	3

Part c

Cells	Technique	°C	Serum dilutions: 1	2	4	8	16	32	64	128	256
A_1			4	4	4	3	3	2	(3)	(2)	–	–
B	Saline	10	4	4	4	4	3	1	(1)	–	–	–
O			3	2	(2)	–	–	–	–	–	–	–
A_1			4	4	4	4	3	2	2	1	(1)	–
B	Bromelin	20	4	4	4	4	3	2	(2)	–	–	–
O			4	3	1	(1)	–	–	–	–	–	–

The Le(a–b–) phenotype of her cells was thought to be false, both because she secreted Le^b substance and because Le^b antigen is commonly absent from the cells during pregnancy (Brendemoen, 1952). However, as her cells still typed as Le(a–b–) when she was not pregnant in 1968, this finding, and the normal secretion of H but weak secretion of Le^b substance noted in her saliva in 1967, suggested, in her, that production of Le^b substance was partially inhibited. Mrs G.C.'s siblings were all group O (Figure 12.2) and their cells were all agglutinated by *Ulex* anti-H lectin to the same titre as the control O cells. Their sera also contained anti-A,B allo-antibodies of normal strength, and no other antibodies were identified in their sera.

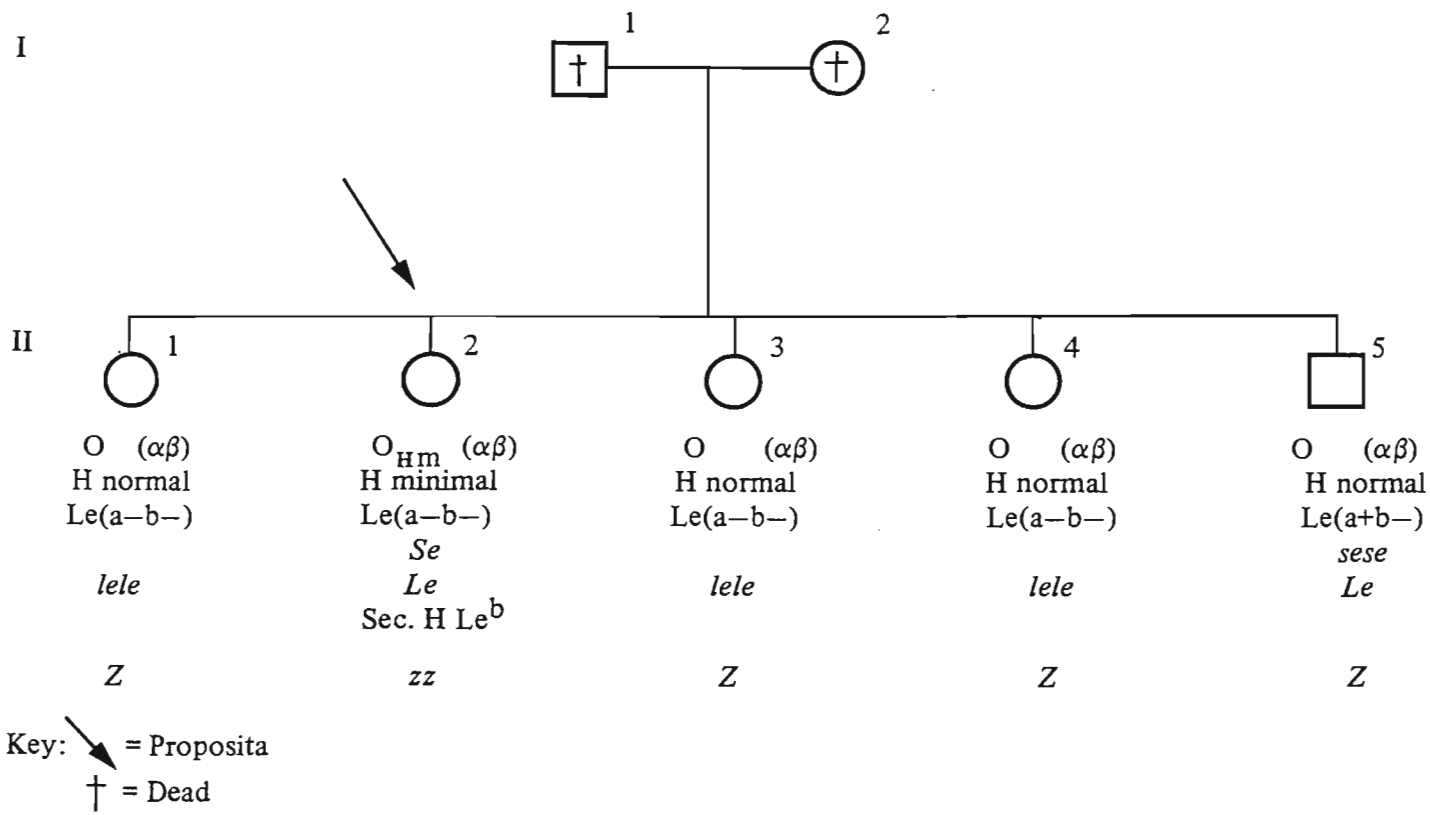
12.5 DISCUSSION

The cells of Mrs G.N., Miss S.M. and Mrs G.G. were identified as para-Bombay, phenotype O^A_{Hm}, O^B_{Hm} and O_{Hm} respectively as, although the groups of their cells appeared in tests with anti-A, anti-B and anti-A,B to be weak group A variant, weak group B variant and O respectively, their cells were not agglutinated by *Ulex* anti-H lectin and were only weakly agglutinated by anti-A+B+H. In addition, the two women and the child were all secretors of ABH substances, and their sera, which contained anti-B, anti-A and anti-A,B respectively, had been found to contain weak IgM cold antibodies that agglutinated all the O cells with which they were tested. The cold antibody in the serum of Mrs G.N., which was surprisingly seen to be inhibited by H substance, appeared to be anti-H, the cold antibody in the serum of Miss S.M. might have been either anti-H or anti-HI, and the cold antibody in the serum of Mrs G.G., was confirmed as being anti-HI. Perhaps the identification of this antibody as anti-H or anti-HI in the para-Bombay's who are secretors depends upon the amount of H antigen present on the owner's cells.

Sringarm, Chupungart and Giles (1972) suggested that O_{Hm} cells were agglutinated weakly by anti-A+B+H because their weakly-expressed H antigen was not recognised by *Ulex* anti-H lectin. In the present study, it was difficult to be certain whether the cells of Mrs G.N. and Miss S.M. had been agglutinated weakly by the anti-A+B+H used, because their cells had weak A or weak B as well as weak H antigen. The cells of Mrs G.N. had been agglutinated by the anti-A+B+H of S. Red. (215), R. Red. (223) and Mrs P.C. (121) less strongly than the cells of Miss S.M., but her cells had also been agglutinated more strongly than those of Miss S.M. by the anti-A+B+H of Mrs P. Gov. (165) (Tables 12.1 and 12.3). If this agglutination was solely due to

Figure 12.2

Family of Mrs G.G., phenotype O_{Hm}



anti-H, the four examples of anti-A+B+H used would have been expected to have reacted equally strongly with the cells of both Mrs G.N. and Miss S.M. The problem might have been resolved if the anti-A+B+H antibodies had been inhibited beforehand with saliva containing H but no A or B substances.

The strong H antigen expressed on the cells of II-2 in the family of Miss S.M. suggested, in addition to a normal Z regulator gene, that II-2 might have inherited a mutant regulator gene, arbitrarily called Z^1 by myself, which coded for excess H antigen on the membranes of her cells. The Z^1 gene would have been inherited by her from her father, I-1 (Figure 12.1), in whom its influence was likely to have been masked by the strong H antigen expected in a group O environment. The gene was not present in her mother, I-2, or in her sister, II-1, as their cells possessed the normally weak H antigen expected in individuals who were group B. Moreover, as the para-Bombay phenotype (and therefore the *zz* genes) of II-3 showed that I-1 was heterozygous Z^1z, the Z^1 gene was clearly capable of expressing itself in single dose. Some support for the existence of a Z^1 gene in the Indians of Natal was provided earlier in this thesis by the 14,52 to 39,56% Indian group B donors who had increased (or 'high') H antigen (Table 3.6, p 38). It was tempting to suggest as well that the A_1 'high' H phenotype recorded in Indians elsewhere (Sathe and Bhatia, 1974) might also be due to the inheritance of a Z^1 gene.

The reduced secretion of Le^b substance and the phenotype Le(a–b–) instead of Le(a–b+) cells in Mrs G.G. suggested that the expected weak *H* and normal strength *Le* gene-specified enzymes in her case might be unable to convert as much precursor substrate as usual into Le^b substance. This situation might have been due either to the limited amount of her H enzyme, or to her *H* gene or her H enzyme malfunctioning in some way. Her limited amount of H enzyme, or her malfunctioning *H* gene or H enzyme, in conjunction with her normal *Le* gene enzyme, might not be able to convert or promote the conversion of precursor glycoprotein substrate into Le^b substance as efficiently as in other people. The situation seemed to be an echo of the Lewis phenotype findings in the parents and children of O_h people, described in Chapter 10, and in the Indian dispermic chimaera shose unusual Lewis phenotype is described in Chapter 14.

No Indians with para-Bombay phenotypes appeared to have been reported before from Natal.

12.6 SUMMARY

Three Natal Indians whose red cells were para-Bombay, phenotype O_{Hm}^{A}, O_{Hm}^{B} and O_{Hm} respectively, have been described. The Indians were all secretors of ABH substances and, as well as their normal ABO allo-antibodies, their sera contained cold antibodies which were identified, in the first Indian as anti-H, in the second Indian as either anti-H or anti-HI and in the third Indian as anti-HI. The family of the Indian whose cells were phenotype O_{Hm}^{B} contained evidence that a mutant regulator gene, Z^{1}, may have caused increased H red cell antigen expression in her family.

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CHAPTER II

THE MNSs SYSTEM

II.1 PAPERS

Notes on some reactions of human anti-M and anti-N sera
Paper 2 by Hirsch, Moores, Sanger and Race

Blood group antigens Mi^a and Vw and their relation to the MNSs system
Paper 3 by Wallace, Milne, Mohn, Lambert, Rosamilia, Moores, Sanger and Race

On the relationship of the blood group antigens Mi^a and Vw to the MNSs system
Paper 6 by Mohn, Lambert, Rosamilia, Wallace, Milne, Moores, Sanger and Race

A comparative study between the S-s-U- phenotype found in Central African Negroes and the S-s+U- phenotype in Rh_{null} individuals
Paper 19 by Vos, Moores and Lowe

Four examples of the S-s-U- phenotype in an Indian family
Paper 20 by Moores

S-s-U- red cell factor in Africans of Rhodesia, Malawi, Moçambique and Natal
Paper 21 by Lowe and Moores

S-s-U- phenotype in South African Negroes
Paper 31 by Hoekstra, Albert, Newell and Moores

S-s-U- phenotype in the Coloured population of Cape Town: problems encountered in paternity tests
Paper 35 by Moores, Marks and Botha

Anti-N in the serum of a healthy type MN person - a further example
Paper 16 by Moores, Botha and Brink

The non-identity of anti-N^A and anti-N in the serum of an MN person
Paper 23 by Booth and Moores

Dantu and MNSs blood groups in a large Natal family
Paper 39 by Moores, Dinnematin and Smart

Anti-Gerbich antibodies and Gerbich-negative Dantu-positive red blood cells in a woman from South Africa
Paper 47 by Moores, de Beer, Marais, Levene, May and du Toit

II.2 INTRODUCTION

The antigens M and N were first described in 1927 by Landsteiner and Levine. The antigen S was identified by Walsh and Montgomery in 1947 and, later the same year, Sanger and Race recognised that S was related to M and N. The first example of anti-s, defining an antigen antithetical to S, was discovered in 1951 by Levine, Kuhmichel, Wigod and Koch. Two rare antigens, Mi^a [Levine, Stock, Kuhmichel and Bronikovsky, 1951] and Vw [van der Hart, Bosman and van Loghem, 1954], which Levine, Robinson, Layrisse, Arends and Sisco [1956] believed were identical, were both seen instead by Cleghorn [1966] to be part of the Miltenberger sub-system. Giles, Chandanayingyong and Webb [1977], and Dybkjaer, Poole and Giles [1981], have since shown that anti-Vw defines Miltenberger Class I, and that anti-Mi^a agglutinates Miltenberger Class I, III, IV and VI red cells.

Anti-U was identified in 1953 and 1954 by Wiener, Unger, Gordon and Cohen. In 1954, Greenwalt, Sasaki, Sanger, Sneath and Race showed that the U- phenotype was S- and s-. The first example of anti-N in a person with N+ red cells was reported by Metaxas-Bühler, Ikin and Romanski in 1961. Anti-Dantu, a previously undescribed antibody for a very low frequency antigen that June Humphreys of Vancouver, Canada, had found but not recorded [1984], was seen in 1980 by Tanner, Anstee and Mawby to react with Ph-variant, and by Unger, Orlina, Dahr, Kordowicz, Moulds and Newman in 1981 to react with N.E.-variant, red cells. These red cells were shown to contain similar but not identical hybrid δ-α sialo-glycoproteins.

Anti-Ge (Gerbich), an antibody for a very high frequency antigen in the Gerbich system, was first described in 1960 by Rosenfield, Haber, Kissmeyer-Nielsen, Jack, Sanger and Race.

II.3 COMMENTARY

II.3.1 Attainments in London, 1955 to 1957

II.3.1.1 M and N antigens and antibodies

In 1928, Landsteiner and Levine noticed that rabbit anti-N was absorbed to extinction with type M red cells but that rabbit anti-M was not absorbed at all with type N red cells. Levine, Ottensooser, Celano and Pollitzer recorded in 1955 that the anti-N lectin, *Vicia graminea*, behaved similarly. In paper 2, studies with four human anti-M and four human anti-N sera confirmed this. The findings were explained by supposing, under certain test conditions, that the molecule of anti-N "fitted" the M site on the red cell membrane, while the molecule of anti-M did not "fit" the N site. The N on type M red cells is now known to be "N" (N quotes) antigen, produced whenever a *S* or *s* gene is present [Issitt, 1985, p345].

II.3.1.2 The antigens Mi^a and Vw

Papers 3 and 6 described the discovery that Mi^a and Vw were two related but not identical low frequency antigens. Approximately 50% of Mi(a+) blood samples were Vw+ and the rest Vw-. No Mi(a-) Vw+ red cells were found. Anti-Mi^a was a relatively common, naturally-occurring antibody. Family studies showed that the genes responsible for the Mi(a+) Vw+ phenotype were usually accompanied by *Ns* and those for the Mi(a+) Vw- phenotype usually accompanied by *MS*.

II.3.2 Attainments in Durban, 1961 to 1991

II.3.2.1 The U- phenotype

Sixteen S-s-U- blood samples from Central African Blacks, approximately twenty-five percent of which were expected to type as U^A or U^B variants [Goldstein and Hoxworth, 1969], four Rh_{null} blood samples (one from the Japanese homozygote and three from persons with homozygous regulator $X^{\circ}r$ genes) and controls were tested with anti-S, anti-s, anti-U and anti-LW [Paper 19]. The results showed that none of the anti-U contained anti-U^A or

anti-U^B. Among the four Rh_{null} samples, the anti-U reacted only with the homozygote. This suggested, when the Rh antigens were absent because two *r* genes had been inherited, that U antigen developed normally. When they were absent because an $X^{o}r$ regulator gene had been inherited, no U antigen was made.

Prior to 1972, the S-s-U- phenotype was thought to be a characteristic of the Black race. Paper 20 showed for the first time that it was also present in the Natal Indians. The physique, facial bone structure, hair colour and texture and skin colour in the Indian family were all typically Indian. Moreover, no evidence was found that the family had other blood group genes or antigens characteristic in Blacks. The Indian S-s-U- phenotype was associated with N. Dosage titrations confirmed that the obligate heterozygotes in the family had single doses of S or s antigen. This supported the view that *u* (or S^u) was an allele of *S* and *s*, rather than that the U- phenotype was the effect of an inhibitor gene independent of the MNSs locus [Race and Sanger, 1958, p99-103]. In tests with an eluate containing potent anti-U and the blood of 1000 Natal Indian and 1000 Natal Black donors, no further U- were detected.

S-s-U- red cells were identified in Zezuru, Karanga, Tonga and Ndebele Blacks of Rhodesia, in Nyanja, Ngoni and Lomwe Blacks of Malawi, and in Chikunda and Sena Blacks of Moçambique [paper 21]. They were not found in a further 12 tribal groups of Rhodesia, seven of Malawi or three of Moçambique or in 15 Zambian and 1000 Natal Black blood donors. One Zezuru and one Ngoni Black had S-s-$U+^W$ red cells, but they were not typed for U^A or U^B. In eight Blacks, the U- phenotype was associated with N, in seven with MN and in two with M. The gene *Nu* therefore predominated. None of the U- Blacks had Rh_{null} red cells.

S-s-U- red cells were also identified in a Black Xhosa woman of Port Elizabeth [paper 31]. Her plasma contained anti-U. The family study showed that two of her five children had this phenotype as well. In antigen dosage titrations, single doses of S or s were confirmed in the obligate family heterozygotes.

Tests with the woman's anti-U and the blood of 1000 further Blacks from the same region yeilded three U-, all of whom denied knowledge of any relationship either with her or each other. In each case, the U- phenotype was associated with N.

Unexpected maternal or paternal exclusions in disputed paternity investigations in Cape Town provided a further rich source of *u* (or S^u) haplotypes [paper 35]. In three of four families and a youth, *u* was accompanied by *N* and in the fourth family by *M*. Dosage titrations with anti-S and anti-s again confirmed that the obligate family heterozygotes had single doses of S or s antigen. The Henshaw+ haplotypes, *MS,He*, *NS,He* and *Ns,He* were also detected in the families.

II.3.2.2 MN with anti-N

A White woman in Port Elizabeth was discovered to have A_1 MSNs red cells and anti-N and anti-H in her plasma [paper 16]. No evidence was found that her N antigen was a sub-type or variant, and autoimmune disease was not detected. The woman's anti-N differed from the anti-N^A of Booth [paper 23], which subdivides Melanesian N antigen into two types, N^A and N^a. Her red cells typed as MN^A.

II.3.2.3 Dantu

During MNSs phenotype and gene frequency studies in Natal Coloured blood donors, the author's habit of routinely including among her reagents an incomplete anti-D used by saline technique proved its worth handsomely! This test had been learnt from Dr Race and Dr Sanger who used it to detect enhanced D antigen. A positive result suggested that an Rh "deleted" phenotype or haplotype was present. When the red cells of one Coloured donor gave a 4+ result, they were immediately re-tested with further reagents. By chance, a sample of anti-Dantu (Cam) was included. Together with a blood specimen from the original Mr Dantu, it had been kindly sent by June Humphreys from Vancouver, Canada, in 1979. A 4+ result strongly suggested that the Coloured donor had Dantu+ red cells

[paper 39]. In the family study, among the 30 co-operative members, 17 more with this phenotype were identified. Their blood was extensively phenotyped, and many carefully-controlled dosage titrations with selected MNSs reagents were made, before the confusing antigens produced by the *Dantu* complex were identified and understood. The complex expressed weak M antigen, visable as such only when an N gene was present *in trans*. When an *M* gene was present *in trans*, titrations with anti-M reagents gave half dose titres and scores. Weak positive or negative results were obtained with *Vicia graminea* anti-N lectin, irrespective of whether or not an *N* gene was present *in trans*. Where a *S* gene was present *in trans*, S antigen was expressed normally. Normal positive, weak positive or negative results were obtained with anti-s, depending upon the reagent used.

The three other gene complexes identified in the Dantu+ family members were *Ms*, *Mu* and *MS,He*. For a time, one member with Dantu+ U- red cells, seven of whose eight children were also Dantu+, was considered a possible *Dantu* homozygote. This was later disproved. Paper 39 warned, when the *Dantu* complex was present in a disputed paternity case, that it would be difficult to assess the genotypes of the participants from their phenotyping results.

Dantu+ red cells were again identified in postnatal studies with the blood of a woman of mixed ethnic origin from Cape Town [paper 47]. The woman had antibodies in her plasma for a high frequency blood group antigen; they were later verified as Gerbich system type anti-Ge3. During phenotyping, the woman's red cells gave an unexpected positive result with another of my routinely-included control sera, which contained anti-S and multiple antibodies for low frequency antigens. Subsequent studies confirmed that her red cells were both Ge:-2,-3 and Dantu+! A sibling had Ge:-2,-3 Dantu- red cells. In the family study, the *Dantu* complex was identified in four generations, and no family members had U- red cells. The proposita's Gerbich and Dantu phenotypes were kindly confirmed by SDS-PAGE analysis by Dr D.J. Anstee (Bristol, UK). No evidence that she

had weakened Kell antigens was detected.

Paper 2

Brit. J. Haemat., 1957, 3, 134.

Notes on Some Reactions of Human Anti-M and Anti-N Sera

W. Hirsch,* Phyllis Moores,† Ruth Sanger† and R. R. Race†

Central Laboratory of the Kupat Holim and Medical Research Council Blood Group Research Unit

In the first detailed account of the discovery of the MN groups Landsteiner and Levine (1928) record how rabbit anti-N sera could be freed of almost all antibody by absorption with M cells, whereas rabbit anti-M sera when absorbed by N cells resisted such non-specific removal of antibody.

The anti-N found in the saline extract of the seeds of *Vicia graminea* has been shown to behave in the same way: Levine, Ottensooser, Celano and Pollitzer (1955) report that M cells treated with the extract, then washed, gave up anti-N on being heated.

As far as we know the present communication is the first study of what is obviously the same puzzling phenomenon shown by human anti-N and anti-M sera. In certain conditions anti-N sera agglutinate M cells while anti-M sera do not react with N cells in any observable way.

The present investigation began when a serum containing a very powerful anti-N agglutinin, specific at 37° C., was found to agglutinate M cells at lower temperatures. This led us to examine more carefully certain human anti-M and anti-N sera available to us.

NOTES ON METHODS

All titrations of serum were made in bulk in saline before distributing 0.01-ml. volumes to the appropriate tubes. The normal packed red cells and the enzyme-treated red cells were suspended in saline in a concentration of 4 per cent. Scores were given to the intensity of agglutination in each tube – their sum made up the titration score. (The scores were +++ = 10, ++ = 8, + = 5, (+) = 3, w = 2, – = 0.)

For each absorption the volume of packed red cells was equal to that of the serum. Before elution, the absorbing cells were washed three times in about thirty times their volume of cold saline; to the washed and packed cells was added half their volume of saline. The mixture was kept at 56° to 58° C. for 5 minutes, and it was then centrifuged in heated cups. A more detailed description of our local variations of these old methods has been given elsewhere (Race and Sanger, 1954).

Temperature

As temperature has played an important part in this work we will explain what we mean by the temperatures we claim.

'3° C.' There was no doubt about this; all the tests – absorptions, centrifugings, titrations, readings of the results, etc. – were done in a room kept at 3° C. All equipment had been there overnight.

'7° C.' This is the average midday temperature of our domestic type of refrigerator in which many of the titrations were incubated. Readings of these tests were made quickly in a

* Postal address: Central Laboratory of the Kupat Holim, P.O.B. 4934, Haifa, Israel.
† Postal address: Medical Research Council Blood Group Research Unit, The Lister Institute, Chelsea Bridge Road, London, S.W.1.

room at about 22° C., on slides brought from the refrigerator in beakers every 3 or 4 minutes. The refrigerator temperature was well maintained in the holes of the wooden blocks holding the tubes, and in the beakers.

'15° C.', '16° C.' and '17° C.' One of these temperatures could quite constantly be achieved, and kept for at least 2 hours, by putting a certain amount of ice into an old incubator detached from its gas supply. Readings were done quickly in a room at about 22° C. on slides taken from the 7° C. refrigerator.

'20° C.', '22° C.' and '23° C.' There was no doubt about these; they were the room temperature of the day.

'37° C.' Our warm incubators are usually between 36° C. and 37° C. Readings were done quickly at 22° C. on slides taken from the incubator in beakers every 3 or 4 minutes.

All the readings were done so quickly that it is difficult to believe that the temperature of the room in which they were done or of the slides could have had much effect.

Treatment of Red Cells with Enzymes

Our methods for treating red cells with enzymes were as follows:

Trypsin. One volume of stock solution of trypsin (50 mg. of 'Trypure Novo', Evans Medical Supplies Ltd., Liverpool and London, dissolved in 5 ml. of 0.05 N-HCl and stored at −20° C.) was added to nineteen volumes of 0.1 M-phosphate buffer of pH 7.7. To four volumes of this solution (diluted freshly each day) was added one volume of washed packed cells and the mixture was incubated at 37° C. for 20 minutes; the cells were then washed once and 4 per cent suspensions were made in saline.

Ficin. One volume of stock solution of ficin (25 mg. of Merck's ficin added to 2.5 ml. of Hendry's buffer (Hendry, 1948) of pH 7.3 and stored for not more than 2 weeks at 4° C.) was added to nine volumes of 2 per cent suspensions of cells in saline, and the mixture was incubated at 37° C. for 15 minutes; the cells were then washed once and 4 per cent suspensions were made in saline. This method was recommended to us by Dr. R. E. Rosenfield.

Papain. One volume of Löw's papain solution (Löw, 1955) was added to seven volumes of 0.1 M-phosphate buffer of pH 7.7. To four volumes of this solution (diluted freshly each day) was added one volume of washed packed cells and the mixture was incubated at 37° C. for 10 minutes; the cells were then washed once and 4 per cent suspensions were made in saline. This way of using Löw's solution was recommended to us by Dr. M. M. Pickles.

SOME REACTIONS OF HUMAN ANTI-N SERA

Four good human anti-N sera were available (Say., Ras., Mic. and Mill.). Besides reacting with N and MN cells, under certain conditions they all reacted with M cells. The strength of the reaction with M cells varied directly with the amount of anti-N in each serum.

A. REACTIONS OF ANTI-N WITH N CELLS

1. *Effect of temperature.* Anti-N agglutinates N cells over a wide range of temperature. The optimum appears to be around 15° C. (Fig. 1).

2. *Effect of enzymes.* Previous treatment of N cells with trypsin, ficin and papain inhibited their reaction with three anti-N sera: Table I shows the effect on the titration scores.

3. *Effect of absorption.* N cells easily remove all antibody from anti-N sera – that for M

cells as well as that for N cells; even the strongest anti-N (Say.) was completely exhausted by two absorptions.

4. *Eluates from absorbing cells.* N cells exposed to anti-N, then washed, gave up anti-N on being heated (Table II). The eluted antibody was very weak compared with that in the original serum. It did not agglutinate M cells.

TABLE I

THE EFFECT OF ENZYME TREATMENT OF RED CELLS ON THEIR INTERACTION WITH HUMAN ANTI-N AND ANTI-M SERA

Interaction	*Serum*	*Titration scores*				*Temperature* (° C.)	*Effect of enzymes*
		Cells untreated	*Cells treated with enzymes*				
			Trypsin	*Ficin*	*Papain*		
Anti-N *v.* N cells	Say.	75	51	14	23	37°	All 3 enzymes much depress
	Say.	71	44	2	49	37°	
	Ras.	44	28	11	30	16°	
	Mic.	34	25	10	7	16°	
Anti-N *v.* M cells	Say.	32	53	32	5	20°	Trypsin enhances, ficin may enhance, papain inhibits
	Say.	42	56	39	13	16°	
	Ras.	12	31	35	5	16°	
	Mic.	0	5	2	..	16°	
Anti-M *v.* M cells	Bes.	40	13	0	19	16°	All 3 enzymes much depress
	W.916	53	5	5	20	16°	
Anti-M *v.* N cells	Bes.	0	0	0	0	16°	No effect
	W.916	0	0	0	0	16°	

For methods of scoring, *see* text.

Check on the efficiency of the enzyme treatment: the M and N cells both had the antigen D, and after treatment they were strongly agglutinated by a weak incomplete anti-D serum but they were not agglutinated by an AB serum.

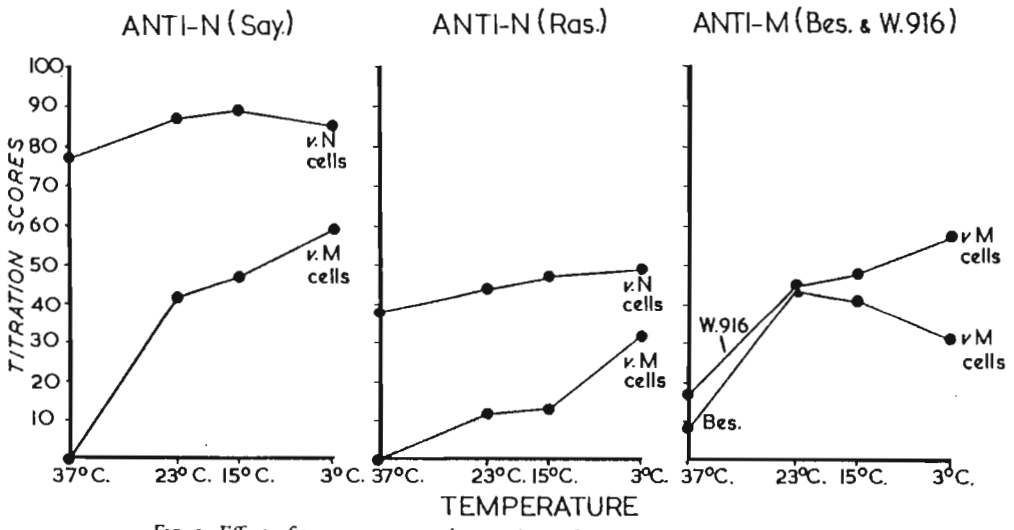

FIG. 1. Effect of temperature on the reactions of certain anti-N and anti-M sera.

B. REACTIONS OF ANTI-N WITH M CELLS

1. *Effect of temperature.* The two strongest anti-N sera did not agglutinate M cells at 37° C. but did so increasingly as the temperature was reduced (Fig. 1). The two weaker anti-N

sera scarcely agglutinated M cells at any temperature, but nevertheless, there must have been interaction because of the elution results (see B4 below) which showed that the M cells must have adsorbed anti-N.

2. *Effect of enzymes.* Here the reaction of anti-N with M cells differs sharply from that of anti-N with N cells (and from that of anti-M with M cells). The reaction of anti-N with M cells is much strengthened by trypsin, strengthened or not affected by ficin and much weakened by papain (Table I).

3. *Effect of absorption.* M cells remove anti-N from anti-N sera; they also remove the antibody in these sera for M cells. Fig. 2 shows the effect of absorbing the two strongest anti-N sera with M cells: it can be seen that the reduction of agglutinin for N cells almost exactly parallels the reduction of that for M cells.

The Ss antigens appear to play no part in this surprising absorption effect: it made no difference whether the absorbing cells were *MSMS*, *MSMs* or *MsMs*.

M cells are nothing like so efficient as N cells at removing the agglutinin from Say. Even twelve absorptions, at 7° C., by M cells did not quite remove all anti-N, though the titration score for N cells was reduced from 100 to 15.

4. *Eluates from absorbing cells.* M cells exposed to four different anti-N sera, then washed, all gave up anti-N on being heated (Table II).

TABLE II

ANTIBODIES ELUTED FROM CELLS EXPOSED TO HUMAN ANTI-N AND ANTI-M SERA

Interaction	*Serum*	*Specificity of eluate*
Anti-N *v.* N cells	Say.	Anti-N
Anti-N *v.* M cells	Say.	Anti-N
	Ras.	Anti-N
	Mic.	Anti-N
	Mill.	Anti-N
Anti-M *v.* M cells	Bes.	Anti-M
	W.916	Anti-M
Anti-M *v.* N cells	Bes.	No antibodies eluted
	W.916	
	Ver.	

Incubation at 3–7° C.; elution at 56–58° C.

SOME REACTIONS OF HUMAN ANTI-M SERA

Four human anti-M sera were available (Bes., W.916, Ver. and Gru.). All of them agglutinated M and MN cells but they did not agglutinate N cells.

C. REACTIONS OF ANTI-M WITH M CELLS

1. *Effect of temperature.* The four anti-M sera reacted weakly, or not at all, with M cells at 37° C., but the strength of the reaction increased as the temperature fell. This was true of three of the sera; the fourth (Bes.) reached an optimum around 20° C. (Fig. 1).

2. *Effects of enzymes.* Previous treatment of M cells with trypsin, ficin and papain inhibited their reaction with two anti-M sera. The effect on the titration scores is shown in Table I.

3. *Effect of absorption.* Anti-M was completely removed from two strong antisera (Bes. and W.916) by a single absorption with M cells.

4. *Eluates from absorbing cells.* M cells exposed to anti-M, then washed, gave up anti-M on being heated (Table II).

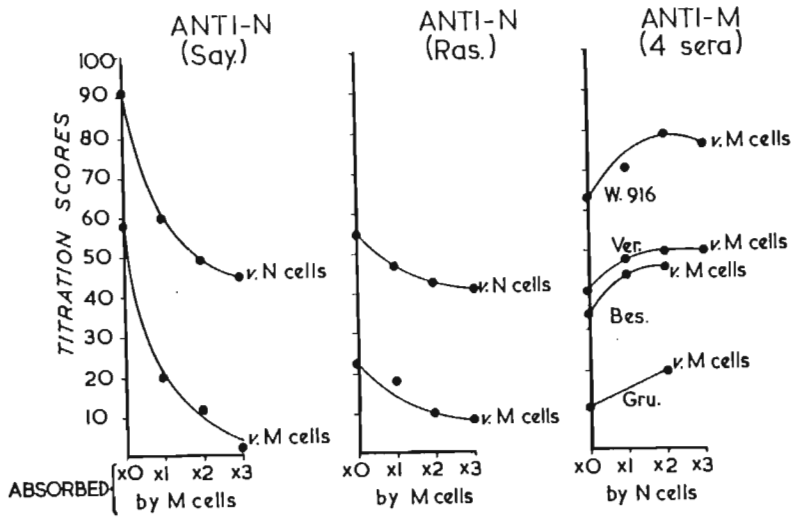

FIG. 2. *Left-hand and centre graphs.* Absorption of two anti-N sera by M cells; the effect is shown on the agglutinin for N cells and on that for M cells.
Right-hand graph. Absorption of four anti-M sera by N cells; the effect is shown on the agglutinin for M cells.
All tests were carried out at 3° C.

D. REACTIONS OF ANTI-M WITH N CELLS

1. *Effect of temperature.* None of the four anti-M sera agglutinated N cells; the temperatures of the tests ranged from 37° C. to 3° C.

2. *Effect of enzymes.* Enzyme treatment of N cells did not make them agglutinable by anti-M (Table I).

3. *Effect of absorption.* Absorption of four anti-M sera by N cells had the rather unexpected effect of increasing the strength of anti-M (Fig. 2). The power of heterologous red cells to revive the reactivity of antibodies in old stored sera was observed by one of us, R. R. R., in 1944; since that time we have frequently made use of this trick. Two of the anti-M sera, Bes. and W.916, used in the present investigation are over 10 years old: all the sera were stored at −20° C.

4. *Eluates from absorbing cells.* No antibody could be detected in eluates from N cells exposed to the three strongest anti-M sera (Table II).

SOME TESTS ON THE RED CELLS OF A BLOOD CONTAINING ANTI-N

The blood of Mr. Say. started the work now being reported; it has in it the most powerful anti-N that we have ever tested. Mr. Say. is a Yemenite Jew, aged 24, who has an enlarged

spleen. He says he has never been transfused. His direct antiglobulin test is negative. His blood groups are:

MNSs system: *MsMs*.

Antisera used: anti-M, -N, -S, -s, -U, -Hu, -He, -Vw, -Mi^a.

Other systems: *OO*; P_1; *CDe/CDe*; *Lu^{b}Lu^b*; *Kk*; Le(a−b+); Fy(a−); *Jk^{a}Jk^a*; Vel+.

Antisera used: anti-A, -B, -H, -'O'; anti-P_1 (lately anti-P), anti-P+P_1 (lately anti-Tj^a) (Sanger, 1955); anti-D, -C, -c, -C^w, -C^x, -E, -e, -E^w, -f, -V; anti-Lu^a, -Lu^b; anti-K, -k; anti-Le^a, -Le^b; anti-Fy^a; anti-Jk^a, -Jk^b; anti-Vel.

In the list of groups we have written genotypes whenever the serological tests disclosed them with certainty or, in the case of *CDe/CDe*, with reasonable probability. Genotypes are printed in italics.

The mother and the two sisters of Mr. Say. are group M; no anti-N could be found in their serum.

Large samples of blood from Mr. Say. and from a control donor of group M were taken within half-an-hour of each other in Haifa. When tested in London 5 days later they were in fine condition, judged by their appearance and by their reactions with many antisera.

TABLE III

REACTIONS OF TWO SAMPLES OF M CELLS, ONE OF THEM FROM A MAN (SAY.) WHO HAS ANTI-N IN HIS SERUM

	Titration scores						
	Human anti-N		*Human anti-M*			*Rabbit anti-M*	
Cells	*Say.*	*Ras.*	*Bes.*	*W.916*	*Ver.*	*No. 155*	*No. 175*
	(17° C.)	(7° C.)	(17° C.)	(17° C.)	(7° C.)	(22° C.)	(22° C.)
Untreated M, Say.	25	2	50	61	18	35	23
Untreated M, control	39	8	48	70	28	39	30
	(17° C.)	(17° C.)					
Trypsinized M, Say.	42	9					
Trypsinized M, control	60	20					

For method of scoring, *see* text.

Reactions with anti-M and anti-N sera. The red cells of Mr. Say. were agglutinated by four rabbit and three human anti-M sera (Bes., W.916, Ver.). His cells were not agglutinated by six rabbit anti-N sera nor by two human anti-N sera (Say. and Ras.) at temperatures at which these sera are specific for N and MN cells. One of the rabbit anti-N sera used is known to agglutinate M^c cells (Dunsford, Ikin and Mourant, 1953), so M^c does not seem to come into the problem.

On the other hand, the red cells of Mr. Say. were agglutinated at 17° C. by the two powerful human anti-N sera (Say. and Ras.) known to agglutinate M cells at temperatures lower than 37° C. It was noticeable that the reactions with the cells of Say. were weaker than those with the control M cells (Table III). This we supposed was due to some 'blocking' of the M antigen of Say. by the antibody in his own serum which is capable of reacting with M cells at any temperature below 37° C.: his cells had been in contact with his own serum at a temperature below 37° C. for 5 days.

If blocking were the true explanation it seemed that the reaction of the cells of Say. with anti-M sera should be weaker than the reaction of the control M cells. On inspecting the results already obtained it was seen that four of the five anti-M sera which had been titrated

had reacted more weakly with Say. than with the control M cells (Table III). The total scores for the five titrations were Say., 187; control M, 215. Moreover, traces of anti-N could be eluted from the washed cells of Say. – further evidence that they were partly blocked.

The serum of Say. was repeatedly absorbed with his own red cells and with those of the control M: both series resulted in the expected parallel fall of antibody for N cells and for M cells (Fig. 2). The absorptive power of the cells of Say. was a little weaker at every step than that of the control M cells – again a result we would expect if his cells were partly blocked. The removal of antibody for Say.'s own red cells was parallel to the removal of antibody for the control M cells; this suggested that the same antibody was acting on both samples of M cells.

DISCUSSION

In the tests reported above anti-M sera did not present any obvious problem: they reacted with cells carrying the antigen M (*see* Sections C1, 2, 3, 4) and they did not react in any observable way with N cells (D1, 2, 3, 4). The anti-M : M interaction was depressed by previous treatment of the cells with enzymes (C2 and Table I). Nor did the reactions of anti-N sera with N cells (A1, 2, 3, 4,) raise any special problem: the interaction was specific, though only at certain temperatures, and it was depressed by previous treatment of the cells with enzymes. But the reaction of anti-N sera with M cells (B1, 2, 3, 4) does demand an explanation. There appear to be two main possibilities:

1. That anti-N sera all have an extra antibody, say anti-x, responsible for their reaction with M cells.

2. That anti-N sera contain but one antibody, anti-N, which can fit the M antigen at certain temperatures or when assisted by enzymes.

1. The hypothetical anti-x would have to share antibody molecules with anti-N, because absorption of anti-Nx sera with Mx cells removes anti-x *and* anti-N equally (B3, 4). The antigen x would have to be very common, for all M cells tested have x.

If x were independent of the MN system, then surely anti-x should be found in anti-M sera, in anti-A, in anti-D, etc., as often as it is found in anti-N sera. In none of these other antisera is there a trace of anti-x but it has been found in all the anti-N sera tested: the antigen x must be part of the MN system.

The absence of anti-x from anti-M sera is shown with the help of enzymes. Enzymes strengthen the x : anti-x interaction of Mx cells and anti-Nx. The interaction of Mx cells and anti-M is inhibited by enzymes (Table I).

We now come to the question whether x can be part of N as well as part of M. Enzyme reactions have given a strong hint that N blood lacks x. On one occasion using untreated and ficinized cells the following titration scores (extracted from Table I) were given by the anti-N serum Ras.:

	Untreated	*Ficinized*
N cells	44	11
M cells	12	35

If N had x then surely the ficinized N cells should have scored as highly as the ficinized M cells.

At this stage of the argument for the existence of anti-x and x we are faced with an insuperable difficulty. If M = Mx and N = N, then why do Mx people make anti-Nx instead of the more reasonable anti-N, and why do N people make anti-M instead of the expected anti-Mx?

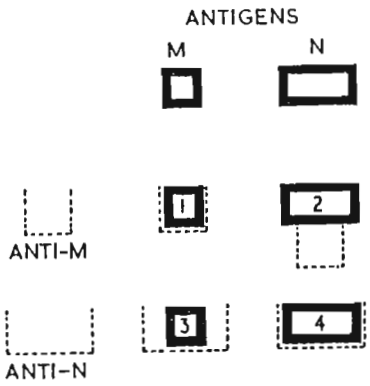

FIG. 3. A summary and possible interpretation of the interactions of anti-M and anti-N with M and N cells.
1 and 4: specific interactions, depressed by enzymes. 2: no interaction. 3: interaction at low temperatures or when assisted by enzymes.

2. We are driven to the other explanation, which is to be preferred in any case because of its simplicity. All the interactions described in this paper can be explained if we assume that anti-N is one antibody, but one which we must allow to combine with M at lower temperatures or when assisted by enzymes; anti-M is denied this heterospecific faculty. Absorption experiments clearly point to there being only one antibody in anti-N serum: absorption with N cells removes all antibody so that the serum no longer reacts with N cells or with M cells; repeated absorption with M cells reduces the reaction for N cells and for M cells equally and step by step.

This explanation, which we think the more likely, can most simply be expressed in a diagram (Fig. 3) which at least is a convenient way of summarizing, and remembering, the rather complicated interactions of anti-M and anti-N sera with M and N cells.

SUMMARY

Four human anti-N and four human anti-M sera have been studied. The anti-M sera did not react with N cells in any observable way. The anti-N sera on the other hand, though specific for N at certain temperatures, did agglutinate M cells when the temperature was reduced or when the cells were treated with enzymes; M cells absorbed anti-N and gave up anti-N on elution.

The simplest explanation appears to be that the anti-N molecule will fit the M molecule when assisted by temperature or by enzymes.

ACKNOWLEDGMENTS

For the anti-M and anti-N sera used in this work we are greatly indebted to the following: Dr. A. Cahan, of New York, Dr. T. J. Greenwalt, of Milwaukee, Miss E. W. Ikin and Dr. A. E. Mourant, of London, Miss Marion Lewis, of Winnipeg and Dr. J. J. van Loghem, Jnr., of Amsterdam.

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Paper 3

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Blood Group Antigens Mi^a and Vw and their Relation to the MNSs System

THE blood group antigen Mi^a was discovered by Levine, Stock, Kuhmichel and Bronikovsky[1] in 1951. Anti-Mi^a, the antibody necessary for the identification of the antigen, was made by a mother in response to immunization by her Mi(a+) fœtus which, as a result, had severe hæmolytic disease. The antigen was shown to be inherited as a dominant character. The antigen was evidently rare, for no example was found in testing 425 random people.

The blood group antigen Vw was discovered by van der Hart, Bosman and van Loghem[2] in 1954. The antibody, anti-Vw, was made by a mother in response to immunization by her fœtus. The father had the antigen and so had several members of his family. The antigen was not present in 740 blood samples from random Dutch people. The antigen was shown to be inherited as a dominant character. Sanger, studying the pedigree, noticed that the gene *Vw* was linked to the *MNSs* locus and was travelling with *Ns*: there were 12 non-cross-overs and no cross-overs.

It was then found[3] that those members of the Dutch family who were Vw+ were also Mi(a+), so it was naturally assumed that Vw was identical with the antigen Mi^a, discovered several years before.

We find that the antigens Mi^a and Vw, though related, are not the same: about half Mi(a+) people are Vw+ and about half are Vw−. No Mi(a−) Vw+ person has been found in several thousand tests on white people.

Though the antigens are rare (the combined incidence of the two phenotypes is one in about a thousand white people) the antibody anti-Mi^a is relatively common and, rather surprisingly, can be present in the serum of people who have not been exposed to either antigen by pregnancy or by transfusion.

From tests on families it has become clear that the gene or genes responsible for both phenotypes, Mi(a+) Vw+ and Mi(a+) Vw−, are linked to the *MNSs* locus. The linkage is of the extremely close type found between the *CDE* genes and between the *MNSs* genes: this was suggested by the absence of cross-overs in the families and confirmed by the observation that in all chromosomes so far analysed the gene or genes responsible for the Mi(a+) Vw+ reaction are accompanied by *N* and *s*, while those responsible for the Mi(a+) Vw− reaction are accompanied by *M* and *S*.

Because of the rarity of the two phenotypes it may be some time before their precise genetic back-

ground is established. We can think of three possibilities. The genes responsible could be alleles: they would fit the CC^w behaviour in the Rh system. (The antigen Mi^a could be like C and the antigen Vw like C^w; anti-Mi^a could be like anti-C, which reacts with Cc and with C^wc, and anti-Vw could be like anti-C^w, which reacts only with C^w.) On the other hand, the genes may be representatives of adjacent loci like *D* and *C* in the Rh system. (The antigen Mi^a could be like D and the antigen Vw like C; anti-Mi^a could be like anti-D+C and anti-Vw like anti-C.) The available genetical and serological details would fit either arrangement; they would not fit so well the third possibility that one gene alone is responsible and that the difference between Mi(a+) Vw+ and Mi(a+) Vw− reflects a position effect exerted by the *Ns* and by the *MS* genes respectively.

The antigens Mi^a and Vw, together with the antigens Hu and He, are demonstrating the complexity of the region of chromosome responsible for the MNSs blood groups and it now appears that at least four loci must be involved.

We are grateful to Dr. Philip Levine, Dr. J. J. van Loghem, jun., and Dr. A. E. Mourant for some of the antisera used in this work. The details of the investigation will be submitted for publication elsewhere.

J. WALLACE
G. R. MILNE

Glasgow and West of Scotland
Blood Transfusion Service,
15 North Portland Street,
Glasgow, C.1.

J. MOHN
R. M. LAMBERT
H. G. ROSAMILIA

Department of Bacteriology
and Immunology,
School of Medicine,
University of Buffalo,
Buffalo 14, N.Y.

PHYLLIS MOORES
RUTH SANGER
R. R. RACE

Medical Research Council
Blood Group Research Unit,
The Lister Institute,
Chelsea Bridge Road,
London, S.W.1.
Dec. 17.

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Paper 6

On the Relationship of the Blood Group Antigens Mi^a and Vw to the MNSs System

J. F. MOHN*, R. M. LAMBERT* AND H. G. ROSAMILIA

Department of Bacteriology and Immunology, School of Medicine, The University of Buffalo, Buffalo, New York

J. WALLACE AND G. R. MILNE

Glasgow and West of Scotland Blood Transfusion Service, Glasgow

AND

PHYLLIS MOORES, RUTH SANGER AND R. R. RACE

Medical Research Council Blood Group Research Unit, The Lister Institute, London

THE DISCOVERY, in 1927, of the MN system of blood groups was the brilliant achievement of Landsteiner and Levine. Subsequent work has entirely confirmed the original findings; it has also shown that the genes and antigens M and N are but part of a complex system. An account of the system as we now understand it will be found elsewhere (Race and Sanger, 1958). The present paper deals with two antigens, called Mi^a and Vw, which are part of the MNSs system but whose precise place in that system remains undecided. When their place is known then will be the time to search for more appropriate symbols.

The antigen Mi^a was discovered by Levine, Stock, Kuhmichel and Bronikovsky in 1951. Anti-Mi^a, the antibody which identified the antigen, was made by a mother in response to immunization by her Mi(a+) fetus which, as a result, had severe hemolytic disease. The antigen was shown to be inherited as a dominant character and it was evidently rare, for no example was found in testing 425 random people.

The antigen Vw was discovered by van der Hart, Bosman and van Loghem in 1954. The antibody, anti-Vw, was made by a mother in response to immunization by her fetus. The father had the antigen and so had several members of his family; it was not present in 740 blood samples from random Dutch people. The antigen was shown to be inherited as a dominant character. Sanger, studying the pedigree, noticed that the gene *Vw* was linked to the MNSs locus and was travelling with *Ns*. As there were 12 non-cross-overs and no cross-over it seemed likely that *Vw* was actually part of the MNSs system and that the linkage was of the very close type first recoginzed in Rh.

It was then found (Levine *et al.*, 1956) that those members of the Dutch family who were Vw+ were also Mi(a+), so it was naturally assumed that the antigens Mi^a and Vw were identical. But, in a preliminary communication, the present

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authors (Wallace *et al.*, 1957) stated that the antigens were not identical: about half Mi(a+) people were Vw+ and about half were Vw−. Furthermore, in all the families studied at that time, in which the segregation was clear, the gene or genes responsible for the Mi(a+) Vw+ reaction were travelling on an *Ns* chromosome, while those responsible for the Mi(a+) Vw− reaction were travelling on an *MS* chromosome.

In the present paper we give details of these families and of others more recently tested, together with the results of tests on random white people.

The investigation began when, almost simultaneously, examples of anti-Mi^a were found in Buffalo and in Glasgow. In Buffalo the antibody was immune and had been the cause of hemolytic disease; the clinical and serological aspects of the case are being reported by Mohn, Lambert and Rosamilia (1958). In Glasgow two examples of anti-Mi^a were found, one in an anti-E serum and the other in an anti-D+C+E serum which unexpectedly reacted with a *cde/cde* member of the staff (in both the anti-Mi^a appeared to be 'naturally occurring' and in both it so happened that anti-Wr^a was also present).

FREQUENCIES

In order to get some idea of the frequency in whites of the two phenotypes, Mi(a+) Vw+, and Mi(a+) Vw−, the blood of nearly four thousand people in Glasgow and in London was tested with anti-Mi^a. The London samples were all tested with anti-Vw as well (the serum having been kindly provided by Dr. van Loghem). When Mi(a+) samples were encountered in Glasgow they were sent to London where they were further tested with anti-Vw. The results are shown in table 1. Large numbers of samples from whites and negroes have also been tested in Buffalo; these are yet to be analyzed and will be the subject of another paper.

None of the nine positive people of table 1 were known to be related to each other. Though the positives are few it seems that Mi(a+) people are more often Vw− than Vw+. It looks as if the incidence of Mi(a+) may be one in about 430 white people.

No examples were found of blood giving the reaction Mi(a−) Vw+; this phenotype could only have been recognized in the 2,071 London samples which were tested with anti-Vw regardless of whether they were Mi(a+) or Mi(a−).

INHERITANCE

The samples for the genetic investigation came from the families of Mi(a+) people in table 1 and from those found in Buffalo. The great majority of the family samples were collected in Buffalo and in Glasgow; they were sent to London where they were further tested with anti-M, anti-N, anti-S, anti-s and anti-Vw.

The family samples were also tested for the A_1A_2BO, P, Rh, Lutheran, Kell, Lewis and Duffy groups; about half of them were also tested for the Kidd groups.

TABLE 1. RESULTS OF TESTS WITH ANTI-Mi^a AND ANTI-Vw ON RANDOM WHITE PEOPLE

	Mi(a+) Vw+	Mi(a+)Vw−	Mi(a−) Vw+	Mi(a−) Vw−	Total
London	2	1	0	2068	2071
Glasgow	1	5	1767		1773
Total	3	6	3835		3844

As a result one extra-marital child was recognized and discounted. No association of the antigens Mi^a and Vw with any of these groups was expected, nor was it found.

Inheritance of Mi(a+) Vw+

The families in figure 1 all show that the phenotype Mi(a+) Vw+ is inherited as a dominant Mendelian character.

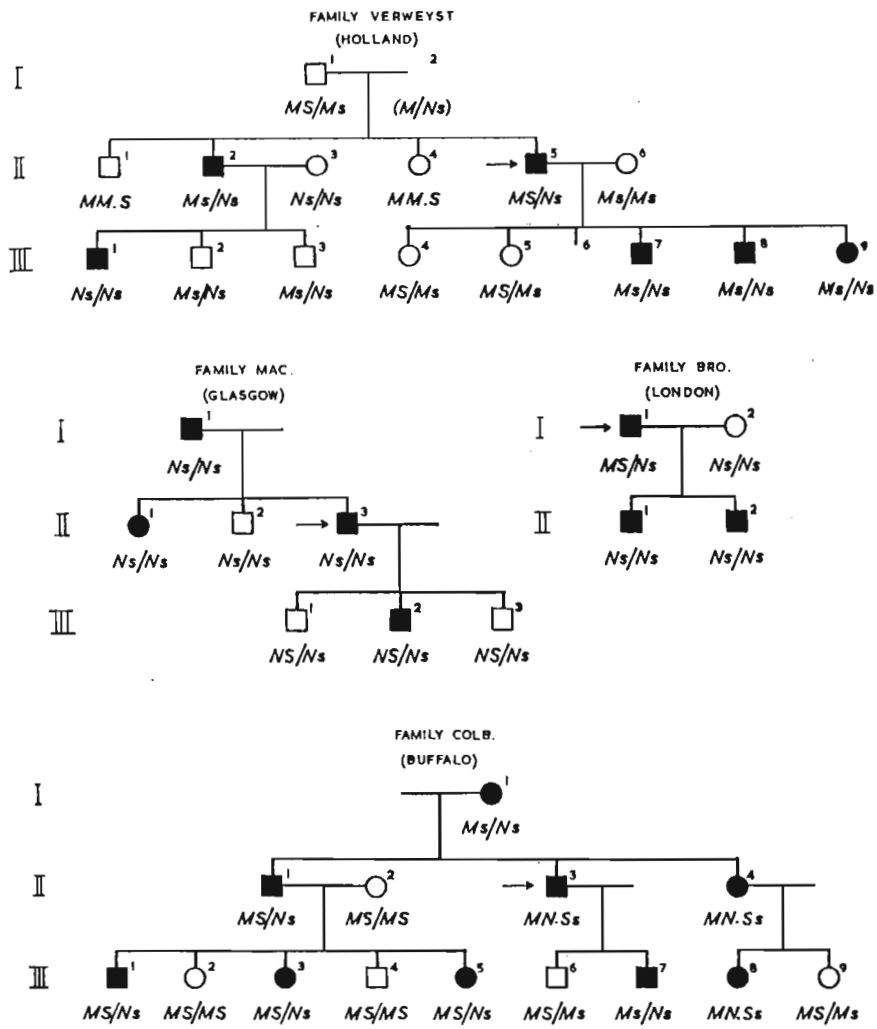

FIG. 1. Families illustrating the inheritance of the phenotype Mi(a+) Vw+. Black = Mi(a+) Vw+: white = Mi(a−) Vw−. Arrow = propositus. In three of the four families (Verweyst, Bro. and Colb.) the gene responsible for the Mi(a+) Vw+ reaction is seen travelling with *Ns*. The family Mac. gives no linkage information.

The groups have been written as genotypes wherever this is disclosed by the serological tests or by the combined evidence of serological tests and the groups of other members of the family. When the groups of missing people have been deduced they are given in brackets. Antisera used: anti-Mi^a, anti-Vw, anti-M, -N, -S, -s. Samples from the original Vw family, Verweyst, were kindly sent by Dr. van Loghem. In this family II-1 and II-4 have not been tested with anti-s and III-9 has not been tested with anti-Mi^a or anti-s.

In family Verweyst, the MNSs groups are such that it can be seen that the gene responsible for the Mi(a+) Vw+ reaction is travelling on an *Ns* chromosome which must have come from the late grandmother, I-2. In generation II, the two Mi(a+) Vw+ children have received their mother's *Ns* while the two Mi(a−) Vw− children have received her *M*; in generation III, all four Mi(a+) Vw+ children have received the grandmaternal *Ns* while all four Mi(a−) Vw− children have received a grandpaternal *Ms* or *MS*. Thus, there are 12 non-cross-overs and no cross-over.

In the family Bro., both Mi(a+) Vw+ children have received their father's *Ns*; so the family provides 2 more non-cross-overs.

In family Colb., the MNSs groups are such that only the offspring of II-1 and II-2 give direct evidence of linkage; their three Mi(a+) Vw+ children have received their father's *Ns* while the two Mi(a−) Vw− have received their father's *MS*. Therefore, this family provides 5 more non-cross-overs and no cross-over. (If we applied the linkage knowledge we could write the genotypes of II-3, II-4 and III-8 as *MS/Ns*.)

Family Mac. gives no information about linkage because I-1 and II-3 are both homozygous *Ns/Ns*. But now that the linkage is established this family shows that once again the gene responsible for the Mi(a+) Vw+ reaction is carried on a *Ns* chromosome. Yet another example is provided by a Mi(a+) Vw+ *Ns/Ns* person in table 1 whose family has not been tested.

The families in figure 1, therefore, establish linkage between the gene responsible for the Mi(a+) Vw+ reaction and the *MNSs* genes. They contain in all 19 non-cross-overs and no cross-over. In five unrelated white people the particular alignment of the new gene with the *MNSs* genes has been made clear, and in each case, the new gene is on an *Ns* chromosome. The probability of this apparent preference for *Ns* being due merely to chance is less than one in a hundred, so there is evidently a marked predilection for partners. This shows that the linkage is not of the traditional kind, with more or less frequent crossing-over resulting in equilibrium, but of the special kind found between the *CDE* genes of the Rh system and between the *MN* and *Ss* genes, in which crossing-over must be a very rare event indeed. We presume that other alignments will be found.

The Mi(a+) Vw+ child III-9 in family Verweyst (figure 1) died at birth. As her cells were sensitized with her mother's anti-Vw, the antigen must be well developed before birth (Hart *et al.*, 1954).

Inheritance of Mi(a+) Vw−

The families in figure 2 show that the phenotype Mi(a+) Vw− is inherited as a dominant Mendelian character.

Four of the families, Hut., Col., Pul. and Erm., prove the existence of linkage between the gene responsible for the Mi(a+) Vw− reaction with the *MNSs* genes.

In family Hut., all the Mi(a+) Vw− people possess the *MS* chromosome of I-2. In this family there are 10 non-cross-overs and no cross-over.

The reconstruction of the groups of I-2, who is dead, may be used as an example of the deductive method that frequently has to be resorted to in translating MNSs phenotypes into genotypes.

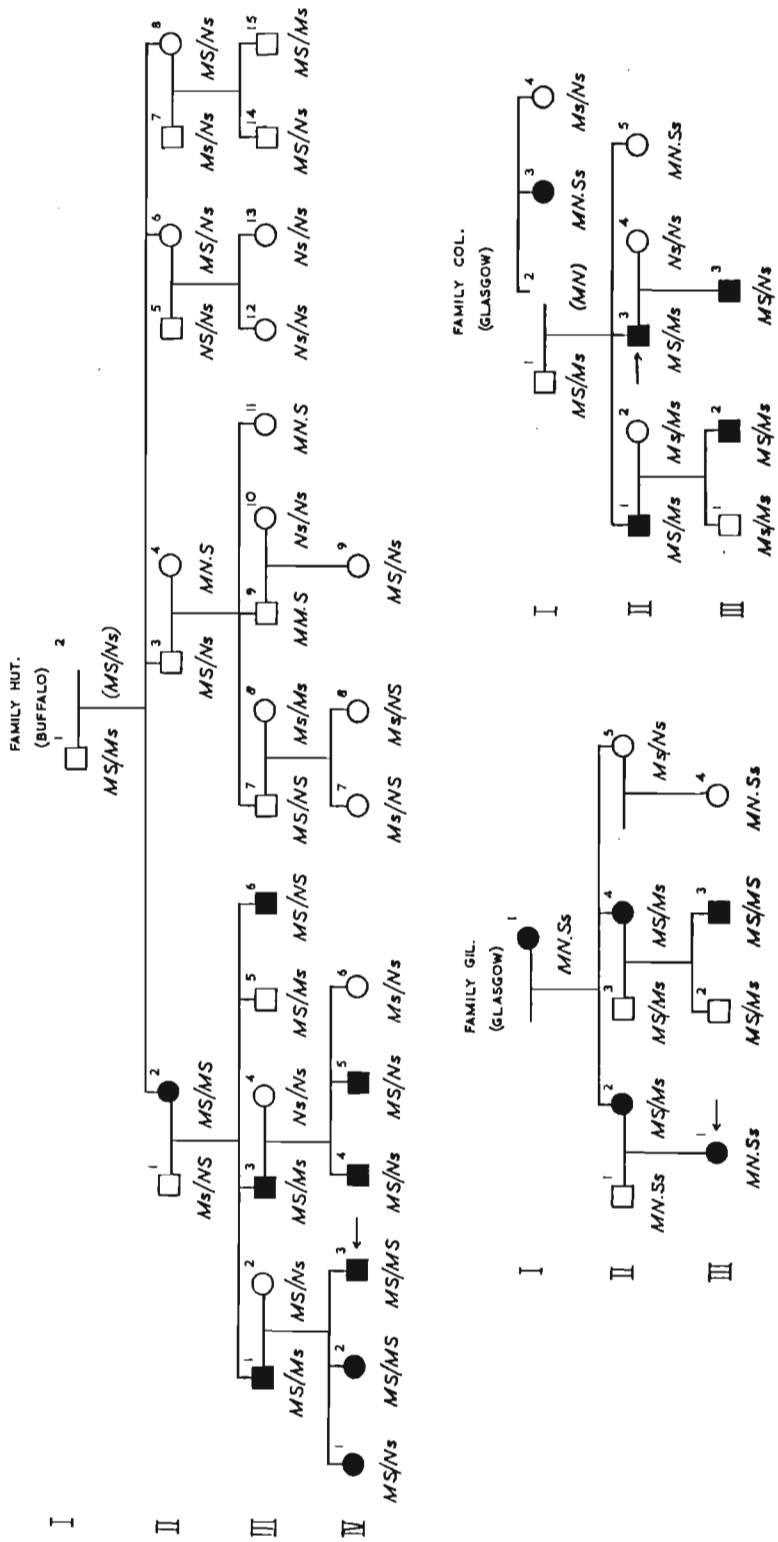
FAMILY HUT.
(BUFFALO)
FAMILY GIL.
(GLASGOW)
FAMILY COL.
(GLASGOW)
I
II
III
IV

FIG. 2.

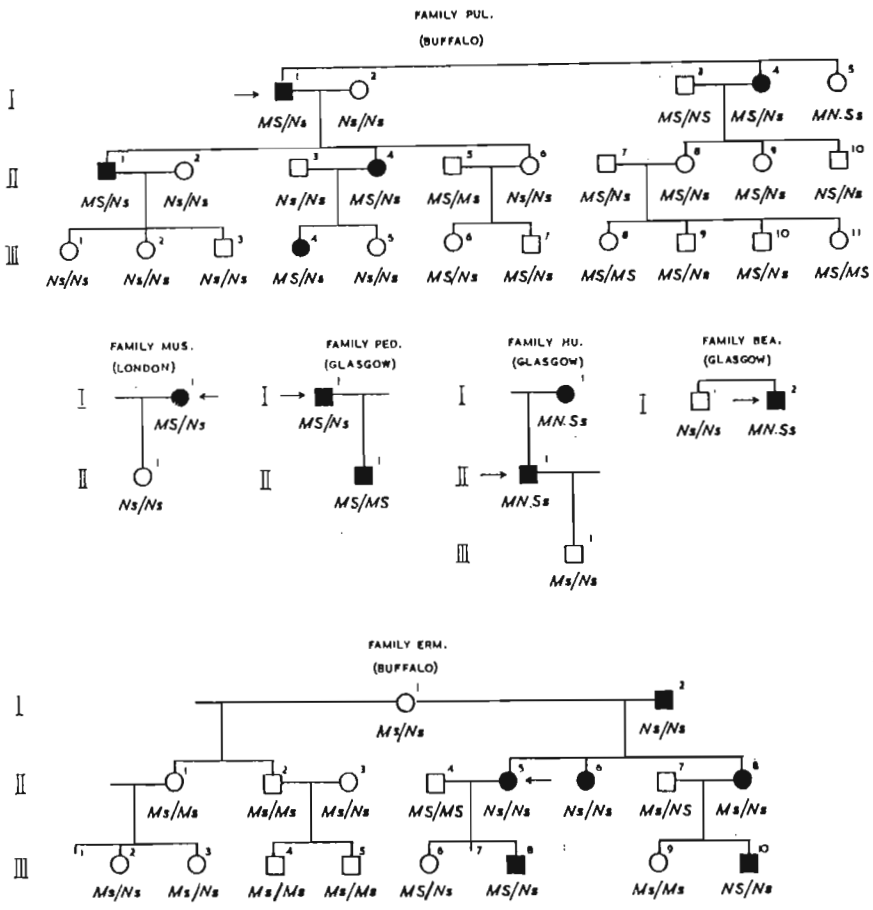

FIG. 2. (*Continued*)

FIG. 2. Families illustrating the inheritance of the phenotype Mi(a+) Vw−. Black = Mi(a+) Vw−: white = Mi(a−) Vw−. Arrow = propositus. In three of the nine families (Hut., Col. and Pul.) the gene for the Mi(a+) Vw− reaction is seen travelling with *MS*. Family Erm., on the other hand, shows the gene travelling with *Ns*. For other notes see legend to Figure 1. In family Hut. II-3, II-4 and their issue were not tested with anti-s.

II-2 is of the genotype *MS/MS*; I-2, therefore, has a chromosome *MS*. III-12 and III-13 have the phenotype Ns; therefore, theis genotypes are *Ns/Ns*; therefore, II-6 whose phenotype is MNSs, must be of the genotype *MS/Ns*; and her *Ns* chromosome must have come from I-2 whose genotype is, therefore, *MS/Ns*. That I-2 has *Ns* may also be deduced if the argument begins with III-15. This illustrates the occasional importance of testing rather unpromising branches of a family which lack the character being studied.

In family Col., again the linkage is with *MS*. We consider that there are 5 non-cross-overs and no cross-over. The argument begins with III-3 but it is rather involved and scarcely worth giving in view of the other overwhelming evidence for linkage.

In family Pul., the linkage again is with *MS* and there are 11 non-cross-overs and no cross-over.

Families Gil., Mus. and Ped. do not give direct linkage information but, knowing that linkage exists, it is clear that in all three families the gene responsible for the Mi(a+) Vw− reaction is again travelling with *MS*.

In family Erm., there are 2 non-cross-overs (III-9 and III-10) and no cross-over. The family is of special interest because it shows that the gene responsible for the phenotype Mi(a+) Vw− can travel with *Ns*, though the usual alignment is evidently with *MS*.

The families of figure 2, therefore, establish linkage between the gene for the Mi(a+) Vw− reaction and the *MNSs* genes. The families contain a total of 28 non-cross-overs and no cross-over.

The *MNSs* chromosome on which the gene for the Mi(a+) Vw− reaction is travelling has been identified in seven unrelated people; in six it is *MS* and in one *Ns*. Since only 25 per cent of all white chromosomes are *MS* here again there is evidently a marked predilection for partners.

The Mi(a+) Vw− reaction is well developed at birth (Mohn *et al.*, 1958). In figure 2, family Hut., IV-3 and IV-5 were tested as cord samples. Presumably the antigen also is developed long before birth because IV-3 suffered from hemolytic disease due to anti-Mi^a. The tests on III-8, family Erm. (figure 2), were also done on a cord sample.

The place of the new genes on the MNSs *chromosome*

This appears to be a difficult problem and it is far from solved. The discussion will be made easier if we are permitted to call the gene responsible for the Mi(a+) Vw− reaction Mi^a and the gene responsible for the Mi(a+) Vw+ reaction *Vw*.

Relation of Mi^a *and* Vw *to the* MNSs *genes*

At least it seems clear that neither Mi^a nor *Vw* are alleles or *M* or *N* or of *S* or *s*. Several Mi(a+) Vw− and Mi(a+) Vw+ people have been found to be of the phenotype MNSs and their M, N, S and s antigens were indistinguishable from normal ones, judged by their reactions with the corresponding antisera.

Since M^c (Dunsford *et al.*, 1953) and M^g (Allen *et al.*, 1958) are alleles of *M* and *N* they could hardly be specially related to Mi^a and *Vw*. Furthermore, samples of blood of the phenotype Mi(a+) Vw− and Mi(a+) Vw+ have been tested with anti-M and anti-N sera capable of detecting M^c—with negative results, and samples have been tested with anti-M^g—also with negative results.

Since S^u is an allele of *S* and *s*, it could hardly be specially related to Mi^a and *Vw*. This is supported by the fact that two S^uS^u people have been tested with anti-Mi^a and three with anti-Vw and found to be negative.

The theoretical possibility that the Mi(a+) Vw− and Mi(a+) Vw+ phenotypes are the result of interaction between the *MNSs* genes appears to be ruled out by the rarity of the phenotypes and by the fact that Mi^a has been identified both on the chromosome *MS* and *Ns*.

Relation to the Hunter and Henshaw genes

The antigens Mi^a and Vw are not the same as the antigens Hu or He. The blood of several white people Mi(a+) Vw+ and Mi(a+) Vw− has been tested with anti-Hu

and anti-He with negative results. The genes *Mi*^{a} or *Vw* could be alleles of *Hu* or of *He*; the rare families that could give information have yet to be found.

Relation to the gene Vr

Neither Mi^a nor Vw is the same as the antigen Vr (Hart *et al.*, 1958). Mi(a+) Vw+ and Mi(a+) Vw− samples have been tested with anti-Vr and were negative. Conversely, anti-Mi^a and anti-Vw did not react with three Vr+ samples from unrelated people. *Mi*^{a} or *Vw* could be alleles of *Vr* but, again, informative families must be extremely rare.

Relation of Mi^a *and* Vw *to each other*

It seems that we must think of *Mi*^{a} as being related to the *MN* genes in the way that *S* or *s* is related to the *MN* genes and the same may be said of *Vw*.

The serological reactions described below do not help to decide whether *Mi*^{a} and *Vw* are alleles of each other like *C* and *C*^{w} in the Rh system or whether they represent closely linked loci like *C* and *D*. Before this can be decided for certain, it seems that a third antibody is required—either anti-Mi^b or anti-vw and, what is more, a family would be needed of a type of such rarity that it would have to have arisen from experimental matings.

Serological evidence

Obvious questions are whether anti-Mi^a contains two antibodies—anti-Mi^a (in a restricted sense) and anti-Vw, and whether anti-Vw contains only one. Attempts to answer these were made by absorbing anti-Mi^a and anti-Vw sera with Mi(a+) Vw+, Mi(a+) Vw− and, as a control, Mi(a−) Vw− red cells. The results are summarized in table 2. The absorptions were done at 20°C. and so were the subsequent titration tests; the cells for these tests were suspended in saline.

Though there appears to be a good deal of cross-absorption, the evidence strongly suggests that the serum Hut. contains two antibodies, anti-Mi^a (in the restricted sense) and anti-Vw. Comparative tests at different temperatures and tests using

TABLE 2. RESULTS OF ABSORBING TWO EXAMPLES OF ANTI-Mi^a AND ONE EXAMPLE OF ANTI-Vw WITH Mi(a+) Vw+, Mi(a+) Vw−, AND Mi(a−) Vw− RED CELLS

Sera		Absorbed by ½ vol. of Packed Cells	Titration Scores: Mi(a+) Vw+ cells	Titration Scores: Mi(a+) Vw− cells	Interpretation
anti-Mi^a (Hut.)	×	Mi(a−) Vw−	60	43	anti-Mi^a + Vw
anti-Mi^a (Hut.)	×	Mi(a+) Vw+	0	15	anti-Mi^a
anti-Mi^a (Hut.)	×	Mi(a+) Vw+ twice	0	11	anti-Mi^a
anti-Mi^a (Hut.)	×	Mi(a+) Vw−	11	0	anti-Vw
anti-Mi^a (Dal.)	×	Mi(a−) Vw−	13	20	anti-Mi^a + Vw
anti-Mi^a (Dal.)	×	Mi(a+) Vw+	0	13	anti-Mi^a
anti-Mi^a (Dal.)	×	Mi(a+) Vw−	0	0	
anti-Vw (Verw.)	×	Mi(a−) Vw−	27	0	anti-Vw
anti-Vw (Verw.)	×	Mi(a+) Vw+	2	0	
anti-Vw (Verw.)	×	Mi(a+) Vw−	27	0	anti-Vw
			Vw	Mi^a	antigens

TABLE 3. THE SOURCES OF CERTAIN ANTI-Mi^a AND ANTI-Vw SERA

Antibody	Donors		Stimulus of Antibody	From
anti-Mi^a	Mrs. Miltenberger	M.S	pregnancy: husband Mi(a+)	Dr. P. Levine
(anti-Mi^a + Vw)	Mrs. Adan.	—	no details	Dr. P. Levine
	Mrs. Hut.	*MS/Ns*	pregnancy: husband Mi(a+) Vw−	Buffalo
	Mrs. Dal.	*Ms/Ns*	no known stimulus	Glasgow
	Mrs. Stev.	*MS/Ms*	no known stimulus	Glasgow
	Mrs. Garl.	*Ms/Ms*	no details	Dr. M. M. Pickles
anti-Vw	Mrs. Verweyst	*Ms/Ms*	pregnancy: husband Mi(a+) Vw+	Dr. J. J. van Loghem
	Mr. Murr.	*MN·Ss*	no known stimulus	Dr. C. Cameron
	Mrs. Bur.	*MN·S*	pregnancy: husband Mi(a+) Vw+	Dr. D. Parkin and Dr. C. Gässer
	1 Donor	*Ms/Ms*	no known stimulus: antibodies found in testing the serum of 703 blood donors	Dr. R. A. Zeitlin
	2 Donors	*MN·Ss*		
	4 Donors	*Ms/Ns*		
	1 Donor	*Ns/Ns*		

different media for the suspension of the red cells (Mohn *et al.*, 1958) give independent support for the presence of two antibodies in serum Hut. That we were not able to achieve an isolation of the anti-Vw in the serum Dal. was due, we suppose, to cross-reaction and to the initial weakness of the antibody.

Anti-Vw, on the other hand, apparently contains but one antibody.

If this interpretation of the antibodies be correct, then only two antigens are needed to fit the pattern of interactions—Vw for blood reacting Mi(a+) Vw+ and Mi^a for blood reacting Mi(a+) Vw−.

Table 3 gives the details, as far as they are known, of the donors of the anti-Mi^a and anti-Vw sera that we have had the opportunity of testing.

If Mi(a+) Vw+ people really have only one antigen, Vw, then we would expect the blood of such people to stimulate anti-Vw free from anti-Mi^a. This is what has happened in families Verweyst and Bur..

If Mi(a+) Vw− people actually possess only one antigen, Mi^a, then we would expect their blood to stimulate anti-Mi^a free from anti-Vw; but, although immunized by a Mi(a+) Vw− fetus, Mrs. Hut. has anti-Mi^a + Vw in her serum (table 2). Indeed, the anti-Vw component in her serum is actually stronger than the anti-Mi^a; but, as the sample was taken in the puerperium, it is possible that the fetus had absorbed enough anti-Mi^a to make the anti-Vw appear the stronger antibody. A sample taken two months later reacted as strongly at 20°C. with Mi(a+) Vw− as with Mi(a+) Vw+ cells suspended in saline.

It is quite possible that the anti-Vw in Mrs. Hut.'s serum was originally present as a "naturally occurring" antibody which probably received some cross-reactive stimulus at the time of her immunization by the antigen Mi^a of her fetus. Anti-Vw is a relatively common antibody; Darnborough (1957) found seven examples in

testing 400 normal sera, and we have confirmed this (see table 3). In our screening of the sera of 703 blood donors, amongst which we found 8 examples of anti-Vw, we disregarded weak reactions and we did the tests at 20°C. More rigorous tests would doubtless have brought to light many more examples of weak anti-Vw.

It is perhaps worth mentioning that in the screening tests the red cells used by Darnborough and by us were of the type Mi(a+) Vw+ which would not be capable of disclosing examples of anti-Mi^a in the restricted sense of the last column of table 2. Such sera would agglutinate Mi(a+) Vw− red cells but not Mi(a+) Vw+ red cells.

Table 3 also shows the MNSs groups, as far as they are known, of the donors of the antisera; they are not in any way remarkable. Darnborough (1957) found that all of the nine donors of anti-Vw were group O. Of the eleven donors of anti-Vw mentioned in table 3, six were A_1, one was A_2, one AB and three were O.

During the course of the work the following "private" antibodies were excluded from being anti-Mi^a or anti-Vw: anti-Di^a, -By, -Wr^a, -Rm and anti-Be^a.

The glimpse they afford of the elaborate structure of the *MNSs* chromosome gives the antigens Mi^a and Vw an interest out of all proportion to their low frequency in the populations so far studied.

SUMMARY

The red cell phenotypes Mi(a+) Vw+ and Mi(a+) Vw− are dominant Mendelian characters. Evidence is produced that each phenotype depends on the presence of a single antigen, Mi(a+) Vw+ reflecting the presence of the antigen Vw, and Mi(a+) Vw− the presence of the antigen Mi^a.

The genes *Vw* and *Mi^a* are part of the *MNSs* complex of genes. In whites *Vw* shows a preference for travelling on an *Ns* chromosome while *Mi^a* shows a preference for travelling on an *MS* chromosome.

The genes *Vw* and *Mi^a* are not alleles of *M* or *N* or of *S* or *s*, whether they are alleles of each other, or of *Hu*, *He* or *Vr* is left in doubt and may remain a problem for a long time.

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A COMPARATIVE STUDY BETWEEN THE S—s—U— PHENOTYPE FOUND IN CENTRAL AFRICAN NEGROES AND THE S—s+U— PHENOTYPE IN Rh_{null} INDIVIDUALS

by

G. H. VOS*, P. MOORES* and R. F. LOWE†

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Although many examples of S-s-U- bloods have been found in Negroes, this phenotype has so far not been reported in White people. To our knowledge only one example of an S-s-U- blood has been described in an Asiatic family [Moores *et al.*, 1971], indicating that this characteristic is Negroid in origin.

In a study of unrelated Negroes in the United States, Francis and Hatcher [1966] found that about 84 per cent of random Ss negative bloods can be expected to be U negative and the remaining 16 per cent U positive. However, there is no evidence to indicate that Ss positive U negative bloods are a common occurrence except in certain cases of red cells lacking all Rh antigens (Rh_{null} phenotype)[Schmidt and Vos, 1967]. The relationship of this phenomenon to the S,s and U determinants was therefore studied for further information about the reactivity of a number of anti-U and anti-s reagents.

A comparative analysis of anti-U and anti-s reagents against known U negative bloods obtained from Central African Negroes and examples of Rh_{null} blood is described.

MATERIALS AND METHODS

All red cells used had been stored at —20°C in a buffered glycerol-citrate solution. Frozen cells were recovered by Weiner's method [1961] using Visking dialysis tubing. In no instance did the frozen and thawed red cells react differently from fresh cells of the same donor before storage.

The various preparations of antisera used were either obtained from commercial sources or were donations where the selective specificity had been repeatedly confirmed. The presence of the U blood group factor in four samples of anti-U was determined by the indirect antiglobulin test. Similar testing procedures also established the presence of the s blood group factor with the exception of anti-s Dublin donated by Dr. O'Riordan, Dublin. The albumin replacement method was used for this reagent.

S typing of the red cells was performed by the saline method in accordance with the manufacturer's directions. Anti-LW AHA was obtained from a patient suffering from acquired haemolytic anaemia of the warm type. This reagent sensitized known LW-positive red cells by the one-stage ficin method. The guinea pig anti-LW serum was used by the saline testing procedure and is the same reagent that was used in the initial study of the LW (D-like) factor by Levine *et al.* [1962]. The degree of agglutination obtained by the various antisera, as shown in Table I were expressed in the following way: intense agglutination 4; strong 3; moderately strong 2; weak 1; no agglutination 0.

* Natal Institute of Immunology, 149 Prince Street, Durban, South Africa.
† Harari Central Hospital, Salisbury, Rhodesia.

RESULTS AND DISCUSSION

In the determination of the U blood group status of four Rh_{null} individuals it was shown that only one (H.A.) possessed a normal U expression of the red cells (Table I). This observation suggests that the absence of a normal U expression in samples H.H., L.M., and E.N., may be due to differences in the serological specificities of the U blood groups.

Before the discovery of the U variant blood groups U^A and U^B [Brice and Hoxworth, 1965; Goldstein and Hoxworth, 1969], it was generally believed that the absence of blood group U was associated with the absence of the Ss blood group factors. That not all S-s- samples of blood can be regarded as U negative was first reported by Allen *et al.* [1963]. From these findings it can be concluded that at least three serological specificities of anti-U reagents may be recognized. Table II lists the designation of the various anti-U reagents and the U blood group factors determined by them. In constructing this table the following reported reactivities were taken into consideration: (1) those that failed to sensitize S-s- bloods [Greenwalt *et al.*, 1954] and (2) those that reacted with known S-s- bloods. The latter presumably represent anti-U sera possessing two serological specificities that can be separated by absorption and elution procedures to reveal the presence of anti-U^A and anti-U^B [Brice and Hoxworth, 1965].

TABLE II

The SsU blood group phenotypes as determined by reactions with various antisera

Reactions of Antisera:					SsU Phenotype
Anti-S	Anti-s	Anti-U	Anti-U^A	Anti-U^B	
—	—	—	—	—	S—s—U—
—	—	—	+	+	S—s—U^{A+B+}
—	—	—	+	—	S—s—U^{A+}
—	—	—	—	+	S—s—U^{B+}

In the present study we wished to establish the possible existence of anti-U variants in four examples of anti-U by determining their reactivity against 16 known S-s- bloods and four examples of Rh_{null} bloods. Accepting that approximately 25 per cent of S-s- individuals can be anticipated to show the U variant phenotype S-s-$U^{(A+B+)}$ [Goldstein and Hoxworth, 1969] then it is possible that at least three S-s-$U^{(A+B+)}$ bloods could be recognized in this analysis, assuming that some of the anti-U reagents used possessed specificity for anti-U^A and anti-U^B. However, Table I shows that not one of the four examples of anti-U reagents appeared to possess the U variant characteristic, suggesting that the incidence of the U variant antibody specificity is not high.

The results reaffirm that red cells lacking factors S and s can be expected to lack a portion of a structural composition of the U factor. The fact that 25 per cent of S-s-U- bloods are known to possess antigenicity for variants U^A or U^B merely indicates that the U system is probably composed of a mosaic of blood group U specificities, as postulated by Goldstein and Hoxworth [1969]. It can therefore be accepted that some of the 16 S-s-U- bloods examined (Table I) are possible S-s-U^A or S-s-U^B variants.

TABLE I

Red cell agglutination tests with various antisera

SsU Phenotype		Reactivity of Red Cells for Antisera													
		Anti-LW		Anti-S			Anti-s					Anti-U			
		AHA	G. Pig	1172	159	60	385	7	Dubl.	Perth	176	959	31	Mah.	128
Control	S+s+U+	4	4	4	4	4	4	4	4	2	2	4	4	3	4
Control	S—s+U+	4	4	0	0	0	4	4	4	2	2	4	4	4	4
Control	S+s—U+	4	4	4	4	3	0	0	0	0	0	4	4	4	4
2671	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
2538	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
1784	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
9484	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
5816	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
7799	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
4166	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
3836	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
3357	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
4661	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
1458	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
2439	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
Krum	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
Amich	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
Kamla	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
Sara	S—s—U—	4	4	0	0	0	0	0	0	0	0	0	0	0	0
H.A.	S—s+U+	0	0	0	0	0	4	4	4	2	2	4	3	3	4
H.H.	S—s+U—	0	0	0	0	0	4	0	4	2	0	0	0	0	0
L.M.	S—s+U—	0	0	0	0	0	4	0	4	2	0	0	0	0	0
E.N.	S—s+U—	0	0	0	0	0	4	0	4	2	0	0	0	0	0

The observation that some Rh_{null} red cells possessed an aberrant U blood group [Schmidt and Vos, 1967] was again confirmed in this study. However, the aberrant U effect could not be confirmed when the same anti-U reagents were tested against the Japanese example of Rh_{null} blood [Ishimori and Hasekura, 1967], suggesting that this anomaly can be attributed to a difference in the structural composition of the Rh_{null} factor.

To provide further information on the genetic background of the Rh_{null} factor extensive family studies were carried out by a number of investigators [Levine *et al.*, 1965; Ishimori and Hasekura, 1967]. A comparative analysis of these studies revealed some remarkable variations which explained why some but not all Rh_{null} bloods possess an aberrant U blood group.

For family L.M. (Table I) it was confirmed that the LW and CDE negative (Rh_{null}) propositus was able to transmit to her child the full expression of the LW and CDE antigens, which she herself, being homozygous for the Rh_{null} factor, did not show. In the Japanese family the parents of the propositus (H.A., Table I) lacked the complete expression of one set of CDE antigens on an allele, indicating that they were heterozygous for the Rh_{null} factor.

These family studies clearly revealed the existence of two mechanisms that influence the LW and CDE expressions. Among the theories proposed to explain the absence of all Rh antigens on the red cells are (1) chromosomal deletions [Ishimori and Hasekura, 1967] and (2) the existence of an independent amorphic gene X°r which blocks the expression of the Rh genes [Levine *et al.*, 1965]. Figure 1 illustrates a tentative model for the two types of conditions that can lead to Rh_{null} expression.

Another effect associated with the blocked production of Rh precursor substance (Rh_{null} samples H.H., L.M. and E.N. Table I) is that the s blood group factor cannot be revealed in some instances by anti-s reagents. This type of anti-s variation was not evident with the Japanese Rh_{null} blood where chromosomal deletion is proposed to play a role. The inability of some anti-s reagents to sensitize Rh_{null} red

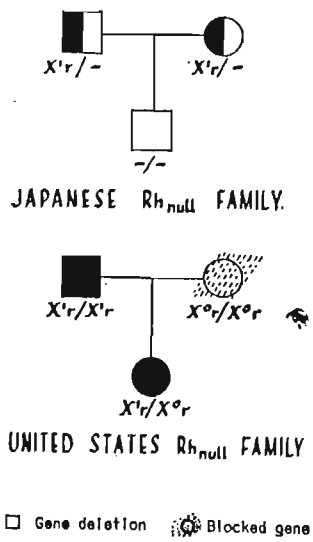

Fig. 1. An illustration of two types of condition that can lead to Rh_{null} expression.

cells was not due to qualitative differences in s specificity. This was evident from a comparative determination of the s antigen value with dilutions of potent anti-s reagents and appropriate control red cells. The observed anti-s anomaly in some Rh_{null} bloods can be pictured as a weakened s antigen expression.

It is difficult to explain the failure of some Rh_{null} red cells (samples H.H., L.M. and E.N.) to react with anti-U reagents. It would appear unlikely that this is due to U variant blood group specificity as the present study clearly indicates.

The fact that the expression of the U antigen can be altered by the same mechanism that is involved in the blocking phenomenon of the Rh precursor substance suggests that the two independent systems Rh and SsU are influenced by a single aberration. Schmidt *et al.* [1967] proposed that this aberration may be due to the sequential action of genes controlling shared terminal sugar(s) which in turn has the capacity to alter the various specificities depending on the basic precursor substance involved [Watkins, 1966]. In the case of the Japanese Rh_{null} blood (H.A.) this hypothesis does not apply because here only the Rh receptors are involved and not U or s determinants of the red cells.

It is significant that the absence of U reactivity can be associated with the presence of s antigenicity. This type of anomaly has so far only been found in some Rh_{null} individuals and therefore cannot be accepted as a characteristic common to Central African U-negative bloods.

SUMMARY

Four examples of Rh_{null} blood and sixteen examples of known S-s-U- red cells were tested against various preparations of S,s and U antisera. Some Rh_{null} red cells were found to have the unusual Ss positive U negative phenotype. This phenomenon could not be explained on the basis of differences in the specificities of the U blood group factor of some Rh_{null} bloods. An alternative possibility may be that the presence or absence of the U blood group factor can be influenced by the actual mechanism which creates Rh_{null} expression. This suggests that chromosomal deletion of all Rh determinants has no effect on the SsU blood groups, whereas the influence of an amorphic gene X°r in the homozygous position, which is known to block all Rh antigen expression, has a definite effect on the SsU blood groups. A review of the genetic background of two Rh_{null} families would seem to be in agreement with this hypothesis.

We wish to thank Drs. P. Levine, P. Sturgeon and H. Hasekura for their generous donations of Rh_{null} bloods. We also wish to thank the Secretary of Health, Rhodesia for his permission to publish the results of this study and Dr. B. G. Grobbelaar, Director of the Natal Blood Transfusion Service, for his continued support.

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Paper 20

Vox Sang. *23:* 452–454 (1972)

Four Examples of the S-s-U-Phenotype in an Indian Family

P. MOORES

The Natal Blood Transfusion Service, Durban

Abstract. Four Natal Indians with the S-s-U- phenotype represent the first recorded examples outside the Negro race. The propositus had anti-c and anti-U in her serum but her CcDee U+ infant was only mildly affected by haemolytic disease of the newborn. There was no consanguinity in the family, and no evidence of Negroid genes.

Dosage studies with anti-S and anti-s showed that only single doses of *S* or *s* accompanied the *u* allele.

The S-s-U- phenotype found in Congo Pygmies, West African Negroes and American Negroes [2], but not so far reported from any other race, is thought to be a Negroid characteristic. In this paper, details will be given of 4 S-s-U- siblings in a family of Natal Indians.

The propositus had 2 normal children and was pregnant for the 3rd time when first seen. She had been given 2 units of blood after her first delivery. Her serum contained an incomplete blood group antibody which agglutinated the red cells of 1,075 Caucasian, 217 Bantu, 298 Indian and 23 mixed race donors. Subsequent investigation showed that her cells were CCDee U- and the antibody was anti-c plus anti-U. At birth, the baby has a strong positive direct antiglobulin test, anti-U and anti-c were eluted from the cord cells but only anti-c was detected in the cord serum. The PCV was 45% and the total bilirubin 5.0 mg per 100 ml. No exchange transfusion or subsequent treatment was required.

The family

The parents had no knowledge of any consanguinity. Two sibs (II 4 and II 11, fig. 1) of the propositus were CCDee U- and were compatible with her

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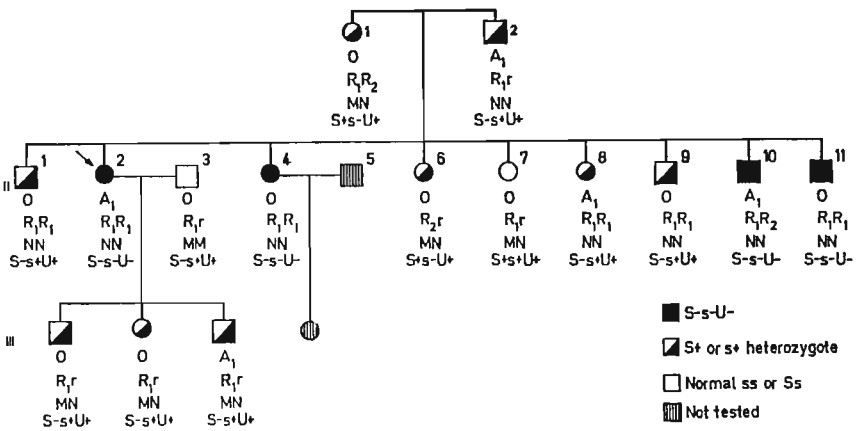

Fig. 1. An Indian family with the S-s-U- phenotype.

serum. Another U- sib (II 10) who was CcDee was not compatible. The remainder of the family blood groups were straightforward, but it was interesting that U- was accompanied throughout by N.

No other essentially Negroid antigens such as strong *Il* [1], weak C, e^8, Henshaw, V or Js(a) [2] were detected in the family, and none of them were cDe, Le(a−b−) or Fy(a−b−) [2]. All were hr^8-positive. Anti-Hunter serum was unfortunately not available for this study.

The family physique, hair texture, skin colour and facial bone shape were all typically Indian. One sib (II 6) had brown instead of black hair and short stubby fingers, but these characters were not considered to be Negroid. There was therefore no physical or serological evidence to suggest Negroid ancestry.

Dosage Studies with Anti-S and Anti-s

Fresh blood samples from the family and *SS*, *ss* and *Ss* controls were drawn on the same day into ACD solution and titrated with 4 anti-S and 2 anti-s sera capable of distinguishing between a single and a double dose of S or s. A sib (II 7), fortunately S+ s+ was a useful additional control within the family.

Table I shows that only single dose results were obtained. This finding supports the view that *u* or S^u is an allele of *Ss*, rather than the effect of an inhibitor gene independent of the MNSs locus.

No further U- was found in more than 1,000 subsequent tests on random Indian blood donors, or in 1,000 local African Negroes.

Table I. Dosage scores with anti-S and anti-s

Generation number	Genotype	Anti-S score	Generation number	Genotype	Anti-s score
I-1	SS^u	3½	II-1	sS^u	4½
II-6	SS^u	3½	II-7	*Ss*	4
II-7	*Ss*	3½	II-9	sS^u	3½
Control	*SS*	6½	Control	*ss*	10
Control	*Ss*	3½	Control	*Ss*	4
Control	*ss*	0	Control	*SS*	0

Acknowledgements

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Author's address: Miss P. MOORES, The Natal Blood Transfusion Service, 149 Prince Street, *Durban* (South Africa)

Paper 21

Human Heredity *22:* 344–350 (1972)

S—s—U— Red Cell Factor in Africans of Rhodesia, Malawi, Mozambique and Natal

R. F. Lowe and Phyllis P. Moores

Harari Central Hospital, Salisbury, and Natal Blood Transfusion Service, Durban

Abstract. 15 examples of S—s—U— have been recorded in Africans of Rhodesia and the adjacent countries of Malawi and Mozambique. None were found in 1,000 Bantu-speaking Negroes of Natal. The frequencies of S—s—U— phenotypes in the various tribes examined are presented, and two possible U variants show that the S—s—U+ phenotype may be present.

Key Words
S—s—U— phenotypes
African population study
Rhodesia
Malawi
Mozambique
Natal

The highest known incidence of the S—s—U— phenotype, 34.9%, was recorded in 1966 by Fraser *et al.* [8] in Efe Pygmies of the north-east Congo and it was suggested that they might form the original reservoir of this phenotype. The reported data showed that the gene was not preferentially associated with either M or N, which is in accordance with the observed evidence among American Negroes [2, 14].

Subsequent studies by Allbrook *et al.* [1] showed that the incidence of the S—s—U— phenotype was 3.14% in Karamojo Negroes (Nilo-Hamites) of Uganda, whilst Cavalli-Sforza *et al.* [5] reported 9.9% in Babinga Pygmies of the western Congo. The frequency in the American Negroes [2, 7, 10], who are known descendants of West African slaves, is 1.2–1.5%.

Rhodesia occupies a position slightly to the south-east of the Congo river basin from which it is separated by Zambia. On the north-east is Malawi, and Mozambique stretches down the entire eastern border. Both Rhodesia and Zambia are on the high central plateau of southern Africa, a geographical position which afforded easy passage throughout history to numerous migrations [15] of African Negroes from East Africa which is adjacent to the eastern part of the Congo. In this study we examine the incidence of the

S—s—U— phenotype in Africans of Rhodesia, Malawi and Mozambique, and in Natal Negroes, all of whom are descendants of the early migratory Negroes of Central Africa.

Materials and Methods

98% of the subjects tested were African females attending the antenatal clinic at Harari Hospital, Salisbury. The remaining 2% were husbands of Rh-negative women attending the clinic, and 25 lepers.

The difficulties of classifying Rhodesian Africans by 'tribe' were described by LOWE [11]. The large linguistic and cultural groupings suggested in that paper have again been adhered to because they are geographically definable. Thus the Barwe, although found in Rhodesia, are generally considered to be Mozambique Africans and have been treated as such, and the Tonga who may be either Rhodesian, Mozambique or Zambian, were in this study considered as Rhodesian because this was their stated birthplace. Rhodesian, Malawi and Mozambique Africans were classified by place of birth and by tribe. Those in whom the replies were not recognisable were classified under 'tribe not known' in table I.

The Natal Negroes were blood donors from the metropolitan and country areas of Natal. The majority were 'Zulus', but the Zulu tribe have been paramount since the early 19th century and absorbed large numbers of other Negro tribes by conquest. They are, therefore, now somewhat heterogeneous. In addition, members of nearby tribes often call themselves by Zulu names when in the vicinity.

Red Cells and Antisera

Clotted blood samples were obtained by venipuncture and were tested within 24 h of collection. The cells were washed twice in normal saline and used at a concentration of 2% against specific anti-U reagents by the indirect antiglobulin test.

The anti-U for this study was prepared by the elution technique of Vos and KELSALL [16] from CCDee U+ cells used to absorb a serum containing anti-c and anti-U. It was diluted 1 in 10 for the initial screening procedure. All negative results were re-examined with the undiluted anti-U eluate and another known anti-U serum. Confirmed U/negative bloods were also tested for their M, N, S and s blood group status.

Results

The S—s—U— phenotype was found in the Zezuru, Karanga, Tonga and Ndebele Africans of Rhodesia; Nyanja, Ngoni and Lomwe of Malawi; and in Chikunda and Sena of Mozambique (table I). It was not found in Natal or Zambia. Although the number of individuals tested in Zambia was very small, their inclusion appeared warranted because the phenotype was frequently detected among small numbers of subjects elsewhere.

Table I. The frequency of S—s—U— in Rhodesian, Malawi, Mozambique and Natal Africans

a Distribution by tribes

Tribe	Country of origin	Observed	
		U+	U—
Karanga	Rhodesia	130	3
Ndebele	Rhodesia	64	1
Tonga	Rhodesia	18	1
Zezuru	Rhodesia	830	6
Budya	Rhodesia	43	0
Korekore	Rhodesia	55	0
Manyika	Rhodesia	165	0
Maungwe	Rhodesia	55	0
Kalanga	Rhodesia	2	0
Ndau	Rhodesia	18	0
Njanja	Rhodesia	6	0
Rozvi	Rhodesia	3	0
Shangana	Rhodesia	22	0
Tawara	Rhodesia	3	0
Venda	Rhodesia	2	0
Not known	Rhodesia	57	0
Lomwe	Malawi	6	1
Ngoni	Malawi	33	1
Nyanja	Malawi	43	2
Chewa	Malawi	47	0
Chawa	Malawi	33	0
Gomani	Malawi	24	0
Manganja	Malawi	7	0
Nguru	Malawi	2	0
Tumbuku	Malawi	3	0
Not known	Malawi	2	0
Chikunda	Mozambique	44	1
Sena	Mozambique	23	1
Barwe	Mozambique	5	0
Pimbi	Mozambique	1	0
Zimba	Mozambique	1	0
Bemba	Zambia	1	0
Lozi	Zambia	1	0
Senga	Zambia	12	0
Not known	Zambia	1	0
Zulu	Natal	1,000	0

Table I (continued)

b Distribution by country (totals)

Country	Observed		Phenotype frequency, %	Observed gene frequency
	U+	U—		
Rhodesia	1,473	11	0.741	0.0861
Malawi	200	4	1.961	0.1400
Mozambique	74	2	2.632	0.1622
Zambia	15	0	0.000	0.0000
Natal	1,000	0	0.000	0.0000

Table II. Details of S—s—U— individuals

Country of origin	Tribe	Group			
		ABO	Rhesus	MN	SsU
Rhodesia	Zezuru	O	ccDee	MN	S—s—U+^{w} (variant)
Rhodesia	Zezuru	AB	ccDee	MN	S—s—U—
Rhodesia	Zezuru	A	D	NN	S—s—U—
Rhodesia	Zezuru	O	D	NN	S—s—U—
Rhodesia	Zezuru	O	ccDee	MN	S—s—U—
Rhodesia	Zezuru	O	D	NN	S—s—U—
Rhodesia	Karanga	B	ccDee	MM	S—s—U— (leper)
Rhodesia	Karanga	O	ccDEE	NN	S—s—U—
Rhodesia	Karanga	B	D	NN	S—s—U—
Rhodesia	Ndebele	B	ccDEe	NN	S—s—U—
Rhodesia	Tonga	O	ccDee	MN	S—s—U—
Malawi	Lomwe	A	ccDee	MN	S—s—U—
Malawi	Nyanja	O	CcDee	MM	S—s—U—
Malawi	Nyanja	A	D	MN	S—s—U—
Malawi	Ngoni	O	ccDee	MN	S—s—U+^{w} (variant)
Mozambique	Chikunda	O	ccDee	NN	S—s—U—
Mozambique	Sena	O	ccDee	NN	S—s—U—

The observed numbers of S—s—U— individuals are presented in table II. From the collective combination it can be seen that there is an increase in the association of the S—s—U— phenotype with the incidence of the N blood group antigen of the MN system. BARKER *et al.* [3] and LOWE *et al.* [12], in a study of Shona and Zezuru Negroes, both showed that the frequencies of M

and N in the general population are about equal, as expected in African Negroes [14].

BRICE and HOXWORTH [4], and GOLDSTEIN and HOXWORTH [9] described the S—s—U+ phenotypes U (A+B—) and U (A—B+) which are collectively known as U variants. Their reactions with anti-U are generally weaker than with other known U+ bloods. Two possible examples of U variants in the present study have been suspected (No 1 and 15, table II) using the criteria previously mentioned. Unfortunately it was not possible to obtain specific anti-U^A or anti-U^B sera to confirm this observation.

Rhesus phenotyping of the S—s—U— individuals using specific anti-C, D, E, c and e reagents revealed no examples of Rh^{null}, some of which may be U— [17].

Discussion

The presence of S—s—U— Negroes in Rhodesia, Malawi and Mozambique has been confirmed. The total absence of the phenotype in Natal Bantu-speaking Negroes is striking and may indicate that their migrating ancestors passed through these territories relatively quickly without absorbing the local inhabitants to any extent, or that it entered the area some time after their departure. Although there is some evidence of a Pygmy-like people and culture in northern Zambia [6] dating from before the Bantu invasions, there are apparently no modern Pygmies as such either there or in Rhodesia now. It is tempting to postulate that during the last 500 years the phenotype may have been obtained from forest Negroes, who live in symbiotic relationship with Pygmies in the Congo and are known to have the S—s—U— phenotype in lower frequency.

A possibility that the Natal Negroes may have lost the S—s—U— phenotype after settling in Natal suggests that it could have a positive selective value north of the Limpopo river, but this seems unlikely considering that it still persists among the American Negroes, who now also inhabit a different environment.

The apparent association of S—s—U— with blood group N is unusual. FRASER *et al.* [8] found no preference for M or N in the Efe Pygmies, while ALLBROOK *et al.* [1] and CAVALLI-SFORZA *et al.* [5] recorded slightly more M than N (table III) in Karamojo Negroes and Babinga Pygmies, respectively. There is no reported association of the N blood group with the S—s—U— phenotype in American Negroes.

Table III. Gene frequencies of M and N associated with S—s—U— in various populations

Populations	Studied by	M	N
Babinga Pygmies	CAVALLI-SFORZA *et al.* [5]	0.191	0.085
Karamojo Negroes	ALLBROOK *et al.* [1]	0.093	0.078
American Negroes	ALLEN *et al.* [2]	0.040	0.048

The presence of possible U variants is in accordance with the work of FRANCIS [7] and ALLEN *et al.* [2] in American Negroes, and of MORTON *et al.* [13] in Brazil. FRANCIS recorded an incidence of 16% U+ in S—s— subjects.

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Authors' addresses: Dr. R. F. LOWE, Harari Central Hospital Laboratory, PO Box ST. 198, Southerton, *Salisbury* (Rhodesia); Miss P. MOORES, Natal Blood Transfusion Service, 149 Prince Street, *Durban* (South Africa)

Paper 31

Vox Sanguinis
Editors-in-Chief: F. H. Allen, L. P. Holländer, M. G. Davey
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Brief Report

Vox Sang. *29:* 214–216 (1975)

S–s–U– Phenotype in South African Negroes

Anneke Hoekstra, A. P. Albert, G. A. I. Newell and Phyllis Moores

Eastern Province Blood Transfusion Service, Port Elizabeth, and Natal Blood Transfusion Service, Durban

Abstract. A Negro woman of the Xhosa tribe of Port Elizabeth in the Eastern Cape was found to be S–s–U– with anti-U in her serum. She had two S–s–U– children, and her husband, father and other children all had single doses of S or s antigen. Three further S–s–U– Negroes were found in a random sample of 1,000 Negro antenatal patients at Port Elizabeth.

The S–s–U– phenotype has not been reported previously in the Negroes of South Africa, although 13 were identified in 1,747 Negroes in Rhodesia, Malawi and Mozambique [1] and 4 in a single family of Indians in Natal [2].

In October 1973, difficulty was experienced in finding compatible blood for a 38-year-old Negro woman of Port Elizabeth in the Eastern Cape. A sample of her blood was sent to Durban where she was found to be S–s–U– with anti-U in her serum.

The proposita, who was expecting her ninth child when her group was discovered, was in need of a blood transfusion for anaemia. She had two sons by her first husband and two daughters by her second husband living; but twins born prematurely at 7 months in her fourth pregnancy, a son aged 1 year in her sixth, and another infant aged 2 weeks in her seventh, had all died without known cause. She had miscarried at 4 months in her eighth pregnancy, but her ninth eventually yielded a normal daughter who did not require treatment of any kind at birth and is still living.

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Family Study

To our surprise, two daughters of the proposita, III 3 and III 9, were also S–s–U– (fig. 1). This indicated that her second husband was probably heterozygous; and comparable blood samples were taken from all the members of the family and from known controls for dosage studies with anti-S and anti-s.

In both Port Elizabeth and Durban, the anti-S and anti-s sera used for the dosage studies were obtained from commercial sources, and previous tests had shown that they were able to distinguish Ss from SS or ss red cells clearly in titrations by the indirect antiglobulin technique. The titrations indicated (fig. 1) that the father of the proposita (I 1), 4 of her 7 sibs (II 1, II 5, II 7 and II 9), and her two eldest children (III 1 and III 2) had single doses of s antigen; and her second husband (II 4), and fourth living child (III 5), had single doses of S antigen. These results lend further support to the view of RACE and SANGER [3] that S^u is an allele of S and s, and not the effect of an inhibitor gene independent of the MNSs locus.

The proposita and her two husbands were not consanguinous. The S^u gene was seen to be inherited in company with an *N* gene in the family, and this was of interest in view of the finding by LOWE and MOORES [1] in 1972 that S^u is more often accompanied by *N* than by *M* in the Negroes of Rhodesia, Malawi and Mozambique. *N* also accompanied

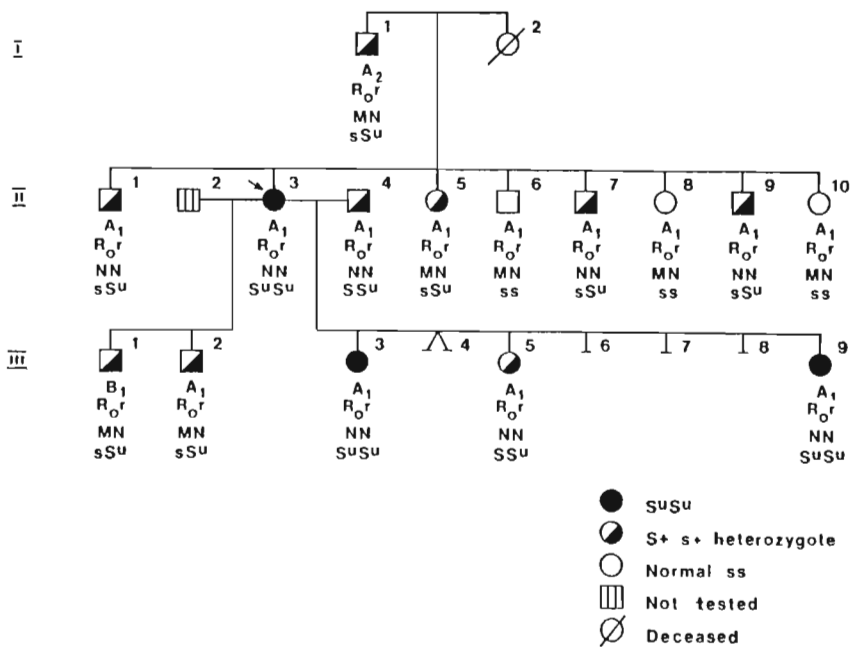

Fig. 1. The inheritance of S^u in a Negro family.

S^u in the Natal Indian family reported by MOORES [2]. The serum of the propositus did not agglutinate S–s+U– Rh_{null} cells, excluding the possibility that she might have anti-s in addition to anti-U as the result of her first two pregnancies.

S–s–U– Phenotype Frequency in the Eastern Cape

A search was made with the serum of the proposita for further examples of the S–s–U– phenotype among 1,000 Negro women who were attending the antenatal clinic at the Livingstone Hospital in Port Elizabeth. Three were found, all of whom were NS^u.NS^u similar to the proposita and her two U– daughters but, on being confronted with one another, they and the proposita denied all knowledge of any relationship.

Historical Background

The proposita and the three random S–s–U– Negroes were all members of the Xhosa tribe which is understood to be descended from the migrant Southern Negroes who entered the Eastern Cape during the 14th century from the north-east and hybridised extensively there with the local Khoikhoi (Hottentots). The absence of this phenotype in the Natal Negroes, whose territory lies to the north-east, and its presence in the Negroes of Rhodesia, Malawi and Mozambique [1], may show that the Eastern Cape and Natal were populated by separate streams of migrants and that the Negroes of the Eastern Cape are more closely related to the Negroes of Rhodesia, Malawi and Mozambique.

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Dr. A. HOEKSTRA, Eastern Province Blood Transfusion Service, PO Box 1567, *Port Elizabeth 6000* (South Africa)

Paper 35

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S--s—U-- PHENOTYPE IN THE COLOURED POPULATION OF CAPE TOWN: PROBLEMS ENCOUNTERED IN PATERNITY TESTS

PHYLLIS MOORES, MELANIE MARKS and M. C. BOTHA

Natal Blood Transfusion Service, P.O. Box 2356, Durban, 4000, and Provincial Blood Grouping Laboratory, Private Bag 4, Observatory, Cape 7935 (South Africa)

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Summary

Two Cape Coloured persons of mixed White, Khoikhoi, Malay, Black parentage in Cape Town whose red cells were found to be S—s—U— establish that the rare S^u allele is present in this population group. The most likely source of the S^u allele in them is the non-indigenous Black slaves who were brought to the Cape in the early years of White settlement there. In two paternity suits involving Cape Coloured persons, maternal and paternal exclusions were present unless the inheritance of an MS^u or an NS^u chromosome was postulated, and *MS*, *NS* and *Ns* chromosomes were seen to accompany *He*+ alleles in them.

Introduction

The rare S—s—U— phenotype, the allele or alleles responsible for which are customarily represented by the symbol S^u, was discovered by Wiener, Unger, Gordon, and Cohen [1, 2] in 1953 in an American Negress. Further investigations showed that the phenotype occurred more frequently in West African than in American Negroes [3], and in 1966 Fraser *et al.* [4] detected it in 34.9% Efe Pygmies in the Congo. Some 15 S—s—U— Central African Blacks were recorded in Rhodesia, Malawi and Mozambique by Vos *et al.* [5] in 1971 and by Lowe and Moores [6] in 1972, and six S—s—U— Southern African Blacks of the Xhosa tribe were found in Port Elizabeth in the Eastern Cape region of South Africa in 1975 by Hoekstra *et al.* [7]. The S—s—U— phenotype has not so far been detected in Southern African Blacks of the Transvaal [8, 9] or Natal [6] Provinces of South Africa or in the Khoikhoi [8] and San [8] peoples, and it has not been recorded before in the Cape Coloured population. However, an Indian family with four S—s—U— members was recorded in Natal by Moores [10] in 1972.

In South Africa the law accepts exclusions of paternity based on the inheritance or non-inheritance by a child of factors in the ABO, MNSs and Rhesus blood group systems. No antibody, however, is known that detects a possible antigen produced by the S^u allele on the red cell membrane. Hence, when S^u in a heterozygous form is suspected in the genotype of one or more of the parties in a paternity suit, following demonstration of an anomalous

inheritance of *S* and *s*, the so far only known way in which this assumption may be supported, other than by the finding of an S—s—U— lineal relative, is by detecting single doses of S or s antigen on the red cells of the participants in Ss antigen dosage titration studies. In three recent paternity suits in Cape Town the results strongly suggested the presence of an S^u allele, and in a random study of reformatory inmates and during a family investigation to find bone marrow donors for a hospital patient, two S—s—U— individuals were found. Both of the latter findings established that the allele S^u does exist in the Coloured population of Cape Town.

Historical background

The Cape Coloured people are the descendants of mixed White, Khoikhoi (Hottentot), Malay (Indian), Black (Negro) parentage dating from the first settlements at Cape Town in the mid 17th century. The Whites came principally from Western Europe, the Malays were slaves imported by the Whites from the Sunda Islands, the Molucca Islands, Ceylon and India [11] at various times until slavery was abolished in 1807, and the Blacks were slaves imported in the 18th century in substantial numbers from Madagascar and the east coast of Africa and a few from Mozambique. In addition, a small party of West African slaves, 170 from Angola and 228 from Guinea, were put ashore at Cape Town in the year 1658 by two ships which subsequently removed 102 of them to Batavia [12]. The San and the indigenous Blacks of South Africa were not taken as slaves, but the Khoikhoi, who were a permanent part of the early Cape scene, were in constant contact with all the newcomers. The Khoikhoi women and the men among the newcomers mixed freely, and the offspring remained with their mothers [12]. In 1972, Botha and Pritchard [12] estimated that the Coloured population at the Cape contained approximately 34% Western European, 36% Southern African and 30% Asian genes.

Materials and methods

The individuals whose blood samples were tested were all members of the Cape Coloured population and were living in Cape Town. Their blood samples were drawn into ACD anticoagulant and were tested within 48 hours of having been received at the Provincial Blood Grouping Laboratory. Cells from the samples were washed thoroughly with saline and 2 - 5% suspensions were prepared in saline for use. The cells were tested with several examples of anti-M, anti-N, anti-S and anti-s, with anti-Henshaw and anti-M^g, and for their P_1, Rhesus, Kell, Duffy and Kidd system factors using commercial blood grouping reagents in strict accordance with the manufacturers' instructions. Known negative and heterozygous or weak positive control cells were included with all the tests. After the results had been assessed, the samples

and controls, to which the red cell preserving fluid of Burgess and Vos [13] had been added, were dispatched immediately to the Natal Blood Transfusion Service in Durban.

At Durban, cells from the blood samples and controls were washed three times with saline and accurately measured 2% suspensions were prepared in the red cell preserving fluid of Burgess and Vos for the Ss antigen dosage titration studies. A set of local control cells was prepared similarly. The anti-S and anti-s reagents used were of both commercial and local manufacture, and the test conditions recommended were adhered to strictly. The reagents were titrated in measured volumes in red cell suspending fluid, and measured volumes of the prepared red cell suspensions were added. After completion of the titrations, the results, which were read both macro- and microscopically, were assessed visually and after having been scored. The MNSs system phenotypes determined in Cape Town were confirmed and the samples were tested with several further examples of anti-M, anti-N, anti-S, anti-s, anti-U and anti-Henshaw, with anti-M^g and anti-M^v, and for their Lutheran and Lewis system factors using commercial and locally standardised reagents. Where necessary, other blood group findings were also confirmed or augmented.

Results

Case 1

The findings (see Fig. 1) showed, unless the inheritance of an S^u allele was postulated, that the woman I 1 and the man I 3 were both excluded as the parents of the child II 1. The MNSs system phenotype of the woman appeared to be MNs, that of the man Ns and that of the child NS. The woman was not excluded as the child's mother by her ABO, Rhesus, Kell and Duffy factors, her leucocyte antigens, her red cell acid phosphatase and her GPT identities, but the red cell acid phosphatase and GPT identities of the man excluded him as the child's father. The Ss antigen dosage titration studies indicated that the man's red cells had a double dose of s antigen, the woman's cells a single dose of s antigen and the child's cells a single dose of S antigen, strongly suggesting that the MNSs system genotype of the man was *Ns/Ns*, that of the woman *Ms/NS*^{u} and that of the child *NS/NS*^{u}. Provided these findings were accepted, the man was further excluded as the child's father by the absence from his cells of the Henshaw factor He+.

Case 2

Figure 2 showed, unless an S^u allele had been inherited, that the woman III 1 was excluded as the mother of the child IV 1 and the woman II 2 was excluded as the mother of the woman III 1. The MNSs system phenotype of the woman II 2 appeared to be Ns, of the woman III 1 MNS and of the child IV 1 Ns. The man III 3 was excluded as the father of the child IV 1 by his different leucocyte antigens, Xg (a+) X chromosome and lack of the

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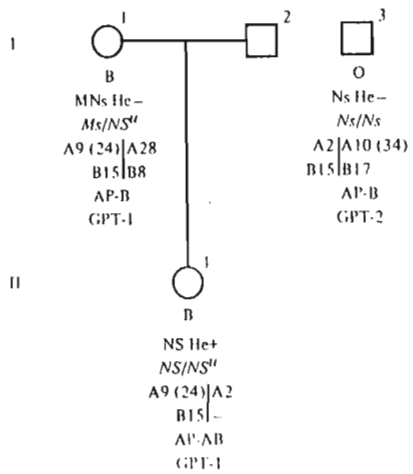


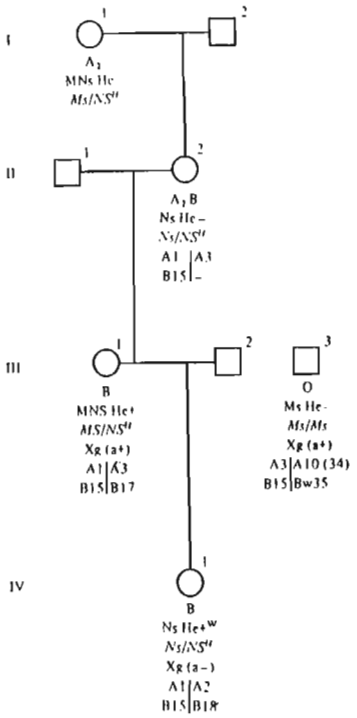


Fig. 1. I 1 is excluded as the mother of II 1 unless an NS^u chromosome is postulated.

Fig. 2. II 1 is excluded as the mother of III 1 and III 1 is excluded as the mother of IV 1 unless an NS^u chromosome is postulated. In this family NS^u is not associated with *He+*.

M^g factor, but the ABO, P_1, Rhesus, Lutheran, Kell, Lewis, Duffy, Kidd and Sd^a factors and leucocyte antigens of the women I 1, II 2, III 1 and of the child IV 1 showed no evidence of any exclusion between them. The Ss antigen dosage titration studies indicated that the red cells of the woman III 1 carried a single dose of S antigen, those of the women I 1 and II 2 and the child IV 1 single doses of s, and those of the man III 3 a double dose of s antigen, strongly suggesting that the MNSs system genotype of III 1 was MS/NS^u, of I 1 Ms/NS^u, of II 2 Ns/NS^u, of IV 1 Ns/NS^u and of III 3 *Ms/Ms*. Provided these findings were accepted, the Henshaw-negative cells of I 1 and II 2 showed that NS^u in the lineal female relatives of IV 1 was not associated with He+ while *MS* in III 1 was associated with this factor. The *Ns* chromosome of the child IV 1 had therefore been inherited from her true father in whom it was accompanied by a different Henshaw-positive allele. The weak Henshaw-positive factor of IV 1 might have been due to the inheritance of a variant *He+* allele from her true father, but the weakness was more likely to be due to antigen immaturity as there was evidence that her P_1, Lewis and I antigens were immature.

Case 3

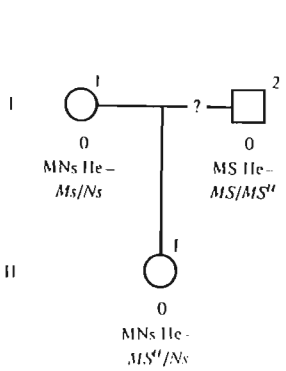
The findings (Fig. 3) showed, unless the inheritance of an S^u allele was postulated, that the man I 2 was excluded as the father of the child II 1. The MNS system phenotype of the woman I 1 appeared to be MNs, that of the man MS and that of the child MNs. However, the man was not excluded as the father of the child according to his red cell ABO, Rhesus, Kell and Duffy factors, and the type MN cells of the child showed that she could not have inherited an M^g or M^k allele from the man. The Ss antigen dosage titration studies indicated that the cells of the woman had a double dose of s antigen, of the man a single dose of S antigen and of the child a single dose of s antigen, strongly suggesting that the MNSs system genotype of the woman was *Ms/Ns*, of the man *MS/MS*^{u} and of the child *MS*^{u}*/Ns*.

Case 4

The proposita in Fig. 4 was an adult suffering from aplastic anaemia and, as part of her treatment, a bone marrow transplant from a family relative had been advised. Four adult relatives were available and their ABO, P_1, Rhesus, Kell, Lewis, Duffy and leucocyte factors were compatible with their identity as siblings of the proposita. Routine tests with MNSs system reagents showed that the red cells of one sibling were phenotype S—s—U—; and further studies, the results of which are shown in Fig. 4, indicated that S^u in them was associated with *N*.

Case 5

In a random study of patients in a Cape Town reformatory, routine tests with MNSs system reagents showed that the red cells of an 18-year-old Cape Coloured youth were phenotype S—s—U—. Further tests established that the youth's genotype was *NS*^{u}*/NS*^{u}, but unfortunately no relatives were available for a family study.



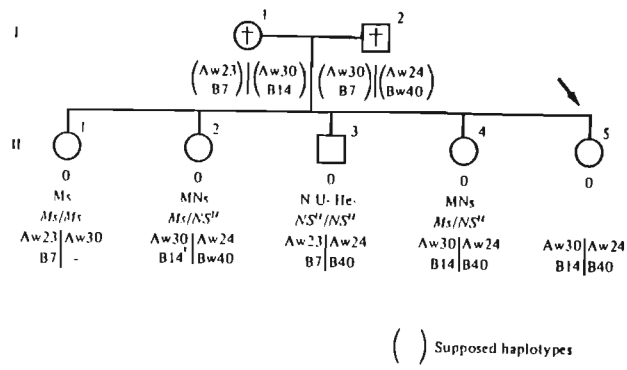


Fig. 3. I 2 is excluded as the father of II 1 unless an *MS*^{u} chromosome is postulated.

Fig. 4. An *NS*^{u} chromosome is present in the family, and II 3 is S—s—U—.

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Discussion

The finding of two Cape Coloured individuals with the S—s—U— phenotype reported in this study confirms that the S^u allele is present in the Coloured population of Cape Town. In both of them S^u was associated with N, and in the individuals of Cape Coloured origin involved in two recent paternity suits in Cape Town, unless an NS^u chromosome had been inherited, the woman was excluded as the mother of the child in one suit and the women in two generations were excluded as the mothers of their respective children in the other. In a third paternity suit, the man was excluded as the father of the child unless the child had inherited an MS^u chromosome from him.

The S—s—U— phenotype has not been recorded in Whites [3] or in the Khoikhoi [8]; the only Indians in whom it has been recorded were four in one family in Natal [10], and the indigenous Blacks (the Xhosa), geographically the closest population group to have the S^u allele, were not living at the Cape in the early years and were not brought there as slaves subsequently [12]. The source of origin of S^u in the mixed White, Malay, Black people known as the Cape Coloureds, therefore, was most likely to be the Blacks of non-indigenous origin, substantial numbers of whom were brought as slaves to the early Cape settlements from Madagascar and the East African coast and a few from Mozambique, Angola and Guinea. The frequencies of MS^u and NS^u have not been recorded in the Blacks of Madagascar, Angola and Guinea [3], but in East African Blacks the frequency of MS^u varies from 3.52% in the Blacks of Mozambique [14, 15] to 13.29% in the Karamojo Blacks of Uganda, and of NS^u from 4.82% in the Blacks of Mozambique [14, 15] to 9.72% in the Karamojo Blacks of Uganda [16]. In the Blacks of Accra and Lagos in West Africa [17] the frequency of NS^u is 12.59% and MS^u has not been recorded.

The presence of S^u in the Cape Coloured people indicates that this allele must be regarded as of very real consequence in all paternity suits involving them in which the putative father is apparently excluded on the basis of his Ss factors. The value of a cautious interpretation of the results of anti-S and anti-s tests in Negroes is well known and has been recorded by Race and Sanger [18] and by Wiener and Wexler [19]. As Ss antigen dosage titration studies are very difficult to perform accurately, even when adequate control red cells and sufficient quantities of anti-S and anti-s able to give clear distinctions between heterozygous and homozygous S— and s— positive red cells are available, we strongly advocate caution in the use of these reagents for this purpose also.

The Henshaw-positive factor, generally regarded as a Negro genetic marker, was found in the Cape Coloured individuals in this study to be associated with MS, NS and Ns. However, Shapiro [9], who found 5 MS, 22 MNS, 14 MNs and 1 Ns Henshaw-positive Cape Coloured individuals in 1000 tested in 1956, showed in family studies that this factor was associated in them most often with Ms. As Jenkins [8] has recorded that *He+* is usually

associated with MS and NS in the Khoikhoi people, and in West African Blacks [3] *He+* occurs most often in association with NS, the most likely source of Henshaw-positive *Ms* and *Ns* chromosomes in the Cape Coloured people was the non-indigenous Blacks of Madagascar, believed to be genetically approximately two parts of Southern African and one part of South-East Asian origin [20], or the Blacks of Mozambique. In Southern African Blacks, Shapiro [8] recorded that *He+* occurs at random with respect to *MS*, *Ms*, *NS* and *Ns* chromosomes.

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Paper 16

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Anti-N in the Serum of a Healthy Type MN Person—a Further Example

P. MOORES, B.Sc., M. C. BOTHA, M.B., CH.B., F.C.PATH.,
AND S. BRINK, M.B., CH.B.

Natal Blood Transfusion Service, Durban, and Provincial Blood Grouping Laboratory, Capetown, South Africa

ABSTRACT

Moores, P., Botha, M. C., and Brink, S.: Anti-N in the serum of a healthy type MN person—a further example. Amer. J. Clin. Path. 54: 90–93, 1970. A healthy, type MN, Caucasian woman, with no evidence of a subgroup of N or autoimmune disease, was found to have anti-N and strong anti-H in her serum. The anti-H in a sample of blood taken after delivery of her fourth child was strong enough to mask the anti-N at 22 C., whereas in pre-delivery samples it had been very weak. The case is similar to one reported by Metaxas-Bühler and associates[2] in 1961, and another by Greenwalt and colleagues[1] in 1966. A family study is presented, and the MN antigen is investigated in detail. The reason for the phenomenon remains unexplained.

METAXAS-BÜHLER and associates (1961)[2] described a healthy male blood donor of type A_1 MN whose serum contained anti-N and a weak anti-H. There were no clinical or serologic signs of autoimmunization and both the M and the N erythrocytic antigens were of normal strength. The anti-N reacted best at temperatures between 12 and 18 C. and agglutinated NN cells much more strongly than MN cells. Above 20 C. type MN cells were either not agglutinated or agglutinated very weakly, while from 0 to 6 C. the anti-H interfered with the reactions, especially when the cells used were group A_2 or O.

In the second example, again a healthy male blood donor (Greenwalt and colleagues, 1966[1]), anti-H was not present and a family study did not reveal any blood group abnormalities.

In this paper details of the case of a third such patient which differs from the preceding two, are given. The propositus was the mother of a small family and at one stage had strong anti-H in her serum.

Report of a Case

A 38-year-old Caucasian woman was pregnant for the fourth time. Her first and third pregnancies had been normal, but the second was an ectopic pregnancy for which she received 4 to 5 units of blood. There had been no previous illnesses. Her cells were typed as MN and her serum contained anti-N. As the anti-N did not agglutinate cells by either the bromelin or anti-

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globulin tests, it was not considered of obstetric significance, and in due course the patient was successfully delivered of an unaffected male infant. Six months later, blood specimens were taken from all members of the family for a detailed examination.

Erythrocytes

The blood groups of the family were:

I_1	O MsNs U+	P−	CDe/cde	(Rh_1rh)	K−	Le(a−b+)	I+
I_2 (the patient)	A_1 MSNs U+	P+++	cde/cde	(rh)	K−	Le(a−b+)	I+
II_1	A_1 NsNs U+	P+++	CDe/cde	(Rh_1rh)	K−	Le(a−b+)	I+
II_3	O MSNs U+	P+++	cde/cde	(rh)	K−	Le(a−b+)	I+
II_4	A_1 MSNs U+	P+++	cde/cde	(rh)	K−	Le(a+b−)	I−

Two commercial anti-M and anti-N sera from rabbits, two commercial anti-M and anti-N plant lectin reagents, and eight anti-M and five anti-N sera from human sources were used to determine the MN groups. Tests were made at 22 C., 12 C., and 4 C. with a 2% saline suspension of cells and were read microscopically after 1 hr. II_1, who had inherited her mother's *Ns*, was expected, assuming it was a subgroup of N, to show weaker results with the anti-N reagents than normal NN controls, while II_3 and II_4 who were the same MN group as their mother but had inherited their *Ns* from their father (I_1), were convenient additional controls within the family.

The results obtained were carefully studied, but no significant differences in M or N antigen strength were detected, and the findings were later confirmed in strictly comparable titrations with the most sensitive of the reagents (Table 1). There was, therefore, no evidence that the N was a subgroup.

Table 1. Titration Results with Anti-N

Cells		Anti-N Dilution									Score
		1	2	4	8	16	32	64	128	256	
Patient	MN	+	+	+	+	±	−	−	−	−	4½
Patient's husband	MN	+	+	+	+	±	−	−	−	−	4½
II_1	NN	++	++	++	+	+	±	−	−	−	8½
II_3	MN	+	+	+	+	±	−	−	−	−	4½
II_4	MN	+	+	+	+	±	−	−	−	−	4½
Control	MM	−	−	−	−	−	−	−	−	−	0
Control	MN	+	+	+	+	±	−	−	−	−	4½
Control	NN	++	++	++	+	+	±	−	−	−	8½

Table 2. Agglutination Reactions of the Patient's Serum with Various Media

	NN (Group O) Cells, Temperature			MN (Group O) Cells, Temperature			MN (Group A_1) Cells, Temperature			Propositus MN (A_1) Cells, Temperature		
	4 C.	12 C.	22 C.	37 C.	4 C.	12 C.	22 C.	37 C.	4 C.	12 C.	22 C.	37 C.
Saline suspension	+++	++	++	+	+	+	±	−	+^{w}	±	−	−	+^{w}	−	−	−
22 % bovine albumin	+	+^{w}	−	−	−	−	−	−	−	−	−	−	not tested			
AB serum	++	+	+	−	+	+^{w}	±	−	not tested				not tested			

Serum

Both samples of the patient's serum agglutinated type NN cells strongly in saline suspensions at 22 C., 12 C., and 4 C., but less strongly at 37 C. (Table 2). MN cells were only just agglutinated at 22 C. and negative at 37 C. NN cells were weakly agglutinated in 22% bovine albumin at 4 C. and 12 C., but not at 22 C. or 37 C., whereas MN cells were not agglutinated in this medium.

In AB serum NN cells were agglutinated at 4 C., 12 C., and 22 C. and MN cells at 4 C. and 12 C. Tests with papain, ficin, trypsin, and bromelin-treated cells were consistently negative.

Anti-H was suspected in the patient's first specimen taken during pregnancy, when group A_1 MN cells were weaker in tests at 4 C. than group O MN cells. In a post-delivery specimen it was surprisingly strong, masking her anti-N at 22 C. completely. Fortunately, there was no difficulty in removing it by inhibition with secretor saliva or blood group-specific substances or by one absorption with group O MM cells.

Absorption with O NN cells at 4 C. removed both the anti-N and the anti-H, whereas absorption with O MM cells left a clear anti-N which agglutinated O MN and O NN cells, but not O MM cells. After absorption with A_1NN cells the serum agglutinated O MN, NN, and MM cells equally well, but did not agglutinate A_1MM cells. In several attempts at elution by various technics neither the anti-N nor the anti-H could be recovered convincingly.

The patient's serum did not agglutinate her own cells at 37 C. or 22 C. (though it did so weakly at 12 C. and 4 C.) and the direct antiglobulin test, using both gamma and non-gamma reagents, was negative.

Anti-N and anti-H were not demonstrated in the sera of other members of the family.

Discussion

No convincing theory for the origin of an anti-N in MN individuals has been put forward so far. Greenwalt and associates (1966)[1] favored a heterologous origin, and we agree that it is theoretically possible for a foreign substance to have properties in common with NN antigen but not MN and to stimulate an "N-like" antibody which would react with NN better than MN cells. However, all three anti-N examples appeared to be naturally-occurring. An "NN-like" protein complex might also be formed by mutation or by an injury leading to loss of natural tolerance in the tissues, but autoimmune disease was not present, unless it was for some reason latent.

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Paper 23

Vox Sang. *25:* 374–376 (1973)

The Non-Identity of Anti-N^A and Anti-N in the Serum of an MN Person

P. B. Booth and Phyllis Moores

Immunohaematology Department, Christchurch Hospital, Christchurch, and Natal Blood Transfusion Service, Durban

Abstract. The anti-N in the serum of an MN person is not anti-N^A, and her red cells are MN^A.

When anti-N^A, which subdivides N of Melanesians and probably of some other racial groups, but not European N, was reported [1], it was suggested that this antibody might be the same as the rare 'non-auto' anti-N found in the serum of certain MN individuals [2, 3, 4, 5]. Comparative material was not available at the time but two further instances of anti-N in MN people were reported subsequently [3, 5], and an investigation has now been made on the South African case (Mrs S). The results detailed below clearly show that the anti-N of Mrs S. is not anti-N^A, and that her red cells are MN^A. Thus, a hoped-for simplification of the MN system has not eventuated.

The anti-N in MN people has not been explained satisfactorily so far. 'Normal' anti-N encompasses the N^A specificity, and it is not unreasonable to suppose that other N sub-specificities exist. Persons lacking one of them may sometimes identify themselves by producing a 'naturally-occurring' antibody which passes for anti-N, but is in reality directed against the missing antigenic specificity possessed by the great majority of the N population. The situation may be analogous to the Gerbich blood group system, where subgroup Ge3 ('Yus' type) persons have so far always identified themselves

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by the production of 'non-Yus' anti-Gerbich (anti-Ge 1, 2), although their cells react with 'Yus' anti-Gerbich (anti-Ge 1, 2, 3). It would seem premature to propose a numerical system for N subspecificities as the investigation of different racial groups, from which information might be obtained, has scarcely begun. Moreover, it has not been established by cross-checking that MN people with anti-N form an homogenous group.

Serological Investigations

Both the anti-N^A (4020) and the anti-N from Mrs S. had become less potent since the original reports. The anti-H, present at one time in the serum of Mrs S, had disappeared. At 4°C, the anti-N^A titre was 4 against cells from Mrs S. and a random European group A_1 MN donor whose blood was collected on the same date. Scores were 17 and 21 respectively. After the anti-N^A serum had been absorbed for 1 h at 20°C with cells from Mrs S., the supernatant scored 19 against NN cells. When absorbed by MN donor cells, the score was 10. An eluate from Mrs S. cells scored 39 and from the MN donor 41 against NN cells. Thus, throughout, the cells of the donor showed marginally greater N^A antigenicity than Mrs S., but this is not considered to be significant.

The anti-N of Mrs S. was tested in parallel with anti-N^A in saline at 4°C with a panel of 45 Melanesian NN cell samples, 20 of which were N^A-negative (N^BN^B). All the samples reacted equally well with Mrs S.

The results show that the N^A-N^B difference is not related to the N antigen of Mrs S. Her cells were tested with four further examples of human anti-N (three from Melanesians), and in every instance the reactions were as expected for an MN sample.

The cells of Mrs S. had normal I^T antigenicity and were Z+ (she is Ss). After 3 weeks' storage at 4°C, their M, N and N^A antigenicity was not found to be reduced.

Acknowledgements

Dr. N. LAHMANN initiated this investigation by drawing the lapsed attention of one of the authors (P.B.B.) to the South African report. The authors are also indebted to Mrs. IRINA BOTHA for arranging the collection of blood from Mrs. S.; to Mrs. S., for her co-operation, and to the staff of the Institute of Human Biology (Director: Dr. R. W. HORNABROOK), Papua New Guinea, for the Melanesian blood samples.

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Request reprints from: Dr. P. B. BOOTH, Immunohaematology Department, Christchurch Hospital, *Christchurch* (New Zealand)

P 8–14

DANTU AND MNSs BLOOD GROUPS IN A LARGE NATAL FAMILY

Phyllis Moores, Hilary Dinnematin and Elizabeth Smart,
Natal Blood Transfusion Service, P.O. Box 2356,
Durban, SOUTH AFRICA.

Dantu is an apparently rare blood group and little has been recorded about it so far. We identified it in a family of mixed White/Southern African Black/East Indian descent in Natal after the red blood cells of a family member blood donor were seen to agglutinate strongly with incomplete anti-D in a saline test.

The red cells of the 17 Dantu-positive family members tested all have reduced M antigen expression. The reaction with anti-M is weakest where an *N* gene has been inherited but apparently genetically *MM* cells give only half dose titres and scores in titrations with this reagent. The Dantu-positive members' red cells also all react unusually weakly with anti-N *(vicia graminea)* lectin. Where *S* has been inherited, the corresponding antigen is expressed normally. The expression of s antigen is either normal or very weak or no s antigen is detected. Where no S and very weak or no s antigen is detected, no U antigen is detected.

The pedigree shows that Dantu is travelling in this family with an *Ms* chromosome. MS^u and *MS, Henshaw-positive*, chromosomes are also present in this family.

The unusual M, N and s antigens of the Dantu-positive family members' red cells caused considerable difficulty in determining the family genotypes. In disputed paternity testing, we believe that similar findings may have implications regarding the correct interpretation of the results.

Abstract: 18th Congress of the International Society of Blood Transfusion, Munich, 1984, p166.

Paper 47

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Anti-Gerbich Antibodies and Gerbich-Negative Dantu-Positive Red Blood Cells in a Woman from South Africa

P. Moores[a], *R. de Beer*[b], *I. Marais*[b], *C. Levene*[c], *R. May*[b], *E. du Toit*[b]

[a] Natal Institute of Immunology, Durban, South Africa;
[b] Provincial Laboratory for Tissue Immunology, Cape Town, South Africa;
[c] Reference Laboratory for Immunohaematology and Blood Groups, Jerusalem, Israel

Key Words. Red cell antigens · Blood groups · Gerbich · Dantu · South Africa

Abstract. Anti-Gerbich type anti-Ge3 antibodies were identified in the serum of a woman of mixed ethnic origin from Cape Town. The woman had type Ge:-2,-3 (Gerbich) red cells on which there was no evidence of weakened Kell antigens. Her red cells were also Dantu-positive.

Since Rosenfield et al. [1] reported in 1960 three antibodies that defined the very common red blood cell antigen Gerbich, rare Gerbich(Ge)-negative red cells have been identified in people of European, Mexican, South American, Indian, Negro and Oriental origin [2]. Two types of Ge-negative red cell membranes, Gerbich and Leach, are known [2]. In the Gerbich type, the sialoglycoproteins β (β-SGP) and γ (γ-SGP), important for maintaining cell shape, are absent. The membranes instead contain an abnormal β-related SGP which apparently fulfils a similar support function [3]. In the Leach type, the β-SGP, γ-SGP and the β-related SGP are absent and the red cells are elliptocytic and/or echinocytic [2]. Ge-negative red cells which give negative results with both anti-Ge2 and anti-Ge3 antibodies are known as the Gerbich type and those giving negative results with anti-Ge2 but positive results with anti-Ge3 as the Yus type. Red cells with Leach type membranes also give negative results with anti-Ge2 and anti-Ge3 antibodies. The Kell and para-Kell antigens are often expressed weakly on Ge-negative red cells of the Gerbich and Leach types but are usually expressed normally on those of the Yus type [4–6]. Anti-Ge2 antibodies are found more often than anti-Ge2,3 antibodies in persons with Ge:-2,-3 (Gerbich) red cells [4].

Dantu is a low-frequency red cell antigen of the MNSs system found so far mainly in people of Negro origin [7]. The membranes of the cells contain a hybrid δ-α form of SGP of which two variants, Ph and NE, are known [7]. The antigens besides Dantu encoded by the *Dantu* gene complex are variable strength M and N, no or very weak U and an unusual form of

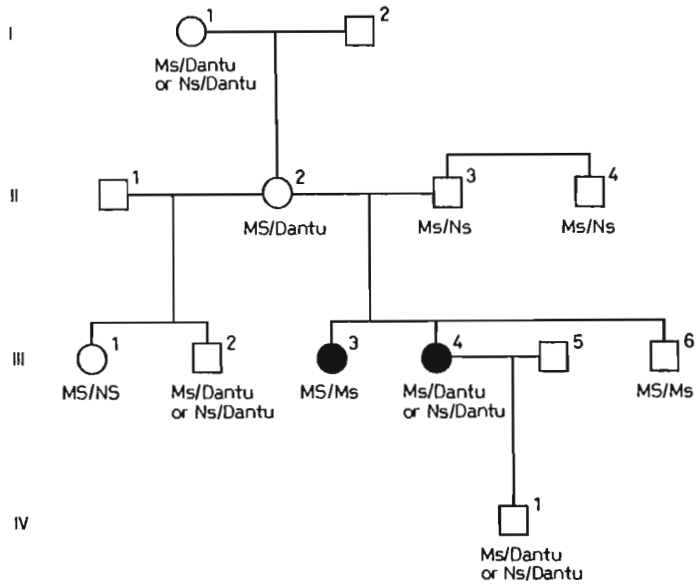

Fig. 1. The family pedigree, showing the probable genes and gene complexes inherited in the MNSs system. Solid circles and squares indicate the members with Ge:-2,-3 red cells.

s that reacts with some but not all anti-s reagents. These results are often masked serologically by the results obtained with the antigens of the normal gene complex inherited in *trans*.

In this paper, studies with the blood of a family of mixed ethnic origin from Cape Town are reported. The proposita had anti-Ge antibodies in her serum; her red cells were both Ge-negative and Dantu-positive. Her red cells had normal strength Kell antigens.

Case History

The proposita (III-4, fig. 1), a primigravida aged 16 years, had group B Rh+ blood. No history of blood transfusion was elicited and no atypical antibodies were detected antenatally in her serum. Her postnatal serum was shown in routine tests to contain atypical antibodies.

The direct antiglobulin test on the cord blood was negative and the baby had group 0 Rh+ blood. The postnatal course was uneventful.

Materials and Methods

Standard serological techniques were used. The antiglobulin reagent contained both anti-IgG and anti-complement components. The phenotyping reagents were used in accordance with the manufacturer's instructions.

Results

The postnatal serum of III-4 reacted with all the red cell samples of an antibody identification panel. The positives were graded 1 to 2+ by both saline technique at ±22°C and indirect antiglobulin technique (incubated at 37°C). In the titrations, titres of 2 and 4–16 were obtained, respectively. By both one-stage bromelin and ficin techniques at ±22°C, the results were inconclusive. When her serum was subsequently tested against a comprehensive panel of red cells negative for high-frequency antigens, three Ge:-2,-3 red cell

Table 1. Results obtained with MNSs and anti-Ge reagents and the family members' red cells

Red cells	M	N	S	s	U	He	Dantu	M_1	Ge2	Ge2,3
I-1	++	++	–	+	+		+			+
II-2	+	++	+	–	+		+			+
II-3	++	++	–	+			–			+
II-4	++	++	–	+			–			
III-1	++	++	+	–			–			
III-2	++	++	–	+	+		+			
III-3	++	–	+	+	+	–	–	–	–	–
III-4	+	++	–	+	+	–	+	–	–	–
III-6	++	–	+	+			–			+
IV-1	+	++	–	+	+		+			

samples were found compatible. The proposita's red cell phenotype was Ge:-2,-3 (Gerbich). As type Ge:-2,3 (Yus) red cells were not readily available, a sample of her serum was sent to Jerusalem, where her antibodies were identified as anti-Ge3.

During phenotyping, an unexpected positive result was obtained with the proposita's red cells and a serum known to include multiple antibodies for low-frequency antigens. In the subsequent studies with monospecific sera and eluates, her red cells were shown to be Dantu-positive. When compared with the MNs control, her red cells reacted with two human anti-M reagents weakly. They gave normal-strength positive results with anti-s and anti-U, rabbit anti-M and anti-N sera and anti-N_{vg} lectins. Negative results were obtained with anti-S reagents.

Table 1 contains the serological results with the family red cells, and figure 1 is the family pedigree. Besides the proposita, III-4, only III-3 had Ge-negative red cells. The *Dantu* gene complex was present in four generations, and no members had U-negative red cells. Although II-2 typed serologically as MNS Dantu+, the *Ms/Ns* genes of II-3 and the *MS/Ms* genes of III-3 and III-6 showed that her most likely genotype was *MS/Dantu.* The two anti-s reagents used clearly had not detected the unusual s antigen encoded by her *Dantu* gene complex. III-4 therefore had either *Ms/Dantu* or, more probably as her cells had strong N antigen *Ns/Dantu* genes. No atypical antibodies were detected in the serum of III-3 and her red cells were compatible with the serum of III-4.

Examination of peripheral blood smears showed no evidence of elliptocytes and/or echinocytes in III-4.

The Kell phenotypes of both III-3 and III-4 were K–k+ Kp(a–b+) Js(a–b+). Titrations with anti-k, anti-Kp^b and anti-Js^b reagents showed that their antigens were comparable in strength with those of the controls.

Dr. D.J. Anstee, Bristol, UK, kindly performed an SDS-PAGE analysis on the

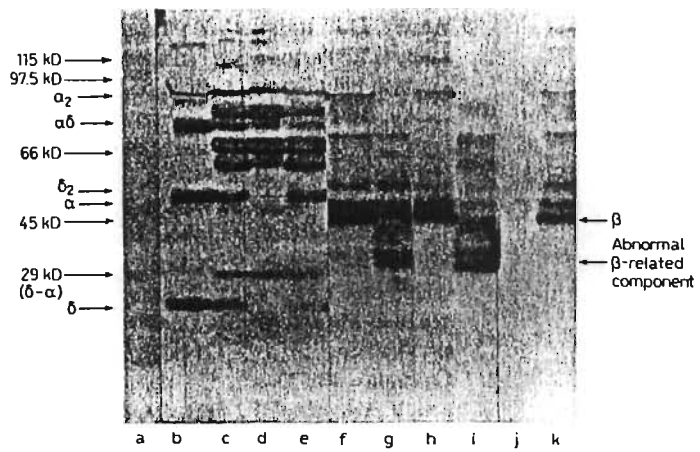

Fig. 2. Results by immunoblot technique using monoclonal antibody R1.3 which reacts with α-SGP, δ-SGP and δ-α^{Dantu}-SGP (tracks b–e) or monoclonal antibody Bric 10 which reacts with β and the abnormal β-protein component of Ge:-2,-3 (Gerbich) red cells (tracks f–k). III-4 shows the patterns expected when the red cells are both Ge:-2,-3 and Dantu-positive. a = Molecular weight markers. Reagent R1.3: b = normal control (Durban); c = III-4; d = Dantu control (Durban); e = Dantu control (Bristol). Reagent Bric 10: f = normal control (Durban); g = III-4; h = Dantu control (Durban); i = Gerbich control (Bristol); j = Leach control (Bristol); k = normal control (Durban).

red cell membranes of III-4. Figure 2 shows the results. The immunoblotting tests with R1.3, a monoclonal antibody which reacts with α, δ and hybrid δ-α^{Dantu}-SGPs, gave the typical bands associated with the Dantu phenotype. The immunoblotting tests with Bric 10, a monoclonal antibody which reacts with β-SGP and the abnormal β-related SGP of Ge-negative red cells, also showed the typical bands associated with the Ge:-2,-3 phenotype.

Discussion

The first South African with anti-Ge antibodies and Ge-negative red cells was identified in 1975. He was Coet., a man of mixed ethnic origin, whose phenotype was Ge:-2,-3 (Gerbich). Daniels [4], who included Coet. in his review, showed that his antibodies had anti-Ge2 specificity.

The anti-Ge3 specificity of the antibodies in III-4 was unusual as anti-Ge2 antibodies apparently occur more frequently in the sera when the red cells are type Ge:-2,-3.

Since the genes for the Gerbich phenotypes are known to be on chromosome No. 2 and those for the MNSs phenotypes on chromosome No. 4, the red cells of III-4 were Ge-negative and Dantu-positive due to the coincidental inheritance of two remarkable rare and interesting genes.

The normal-strength Kell antigens of III-3 and III-4 showed that additional hybrid δ-α^{Dantu}-SGP apparently does not give rise to weak Kell antigens when the red cell membranes also lack β-SGP and γ-SGP.

Acknowledgements

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Phyllis Moores, PhD
Head, Immuno-Haematology Research Laboratory
Natal Institute of Immunology
PO Box 2356
4000 Durban (South Africa)

CHAPTER III

THE P SYSTEM

III.1 PAPERS AND STUDIES

An investigation in Natal Bantu and Indian populations for the presence of anti-Tj^a-like haemolysins
Paper 12 by Moores and Dunning

The P_1H antibody
Unpublished study 8, Ph.D. thesis, p204-221

III.2 INTRODUCTION

The P groups, P+ and P-, were discovered in 1927 by Landsteiner and Levine using anti-P made in rabbits. In 1955, Sanger recognised that anti-Tj^a, an antibody for a very high frequency antigen which had been found by Levine, Bobbitt, Waller and Kuhmichel in 1951, belonged to the P system. P+ and the vast majority of P- red cells were Tj(a+), and the few remaining P- red cells were Tj(a-). The nomenclature was therefore adjusted. P+ became P_1, P- became P_2 and Tj(a-) became p. Anti-P became anti-P_1 and anti-Tj^a anti-PP_1P^K. Rare P^K red cells were identified in 1959 by Matson, Swanson, Noades, Sanger and Race.

Some antibodies for interaction products between genes in the P system and those of the Ii antigens are known. They are anti-IP_1 [Issitt, Tegoli, Jackson, Sanders and Allen, 1968], anti-I^TP_1 [Booth, 1970], anti-iP_1 [McGinniss, Kaplan, Bowen and Schmidt, 1969] and anti-IP [Allen, Marsh, Jensen and Fink, 1974]. Other antibodies for interaction products between genes in the ABO system and the Ii antigens are anti-IA, -IB, -IH, -iH, -IBH and -IAB [Issitt, 1985, p199].

III.3 COMMENTARY

III.3.1 Attainments in Durban, 1961 to 1991

III.3.1.1 Anti-Tj^a-like haemolysin

In 1961, Wren and Vos, and in 1964, Vos, Celano, Falkowski and

Levine described an anti-Tj^a-like haemolysin which was often present for a short period in the sera of women from Perth, Western Australia, who were habitual aborters. In Paper 12, we described the results of our studies with the sera of 128 Black and 70 Indian women in Durban, all of whom were in imminent danger of aborting. No evidence of a similar haemolysin was found. The haemolysin of Wren and Vos was therefore attributed to an unknown factor in the Perth environment.

III.3.1.2 The P_1H antibody

During saline tests at room temperature made to detect unwanted cold antibodies in sera selected for standardisation as anti-A grouping reagents, three sera were found to agglutinate weakly and in mixed-field patterns some but not all the screening cells from group O Blacks [Study 8]. Dr Giles (Blood Group Reference Laboratory, London) kindly offered to help identify the relevant antibodies. Her findings suggested that they were some type of anti-P_1. Later, in Durban, studies showed that the antibodies preferentially agglutinated red cells on which both extra strong P_1 and extra strong H antigen were represented (mainly but not exclusively group O). The corresponding antigen, which may be an interaction product between the the genes for P_1 and H, was temporarily given the name P_1H. In a family study, it was shown to be inherited. Anti-P_1H, a so far unrecorded antibody specificity, was inhibited by P_1 but not by H substance.

Paper 12

AN INVESTIGATION IN NATAL BANTU AND INDIAN POPULATIONS FOR THE PRESENCE OF ANTI-Tj^a-LIKE HAEMOLYSINS

P. Moores and E.K. Dunning

The Natal Blood Transfusion Service

A special haemolysin, subsequently identified as anti-Tj^a-like was reported by Wren and Vos, and Vos et al[3, 2] in 1961 in the population of Perth, Western Australia. This haemolysin is found only in the serum of pregnant women who are habitual aborters, is often present for only very short

periods and disappears altogether as soon as the conceptus has been lost or the pregnancy completed successfully. Specimens must be taken for examination at frequent intervals (1 week) if the haemolysin is to be detected in a particular case but no difficulty was experienced in obtaining a positive result with 45 hour old samples sent in by post. Of 100 cases tested, 39% of the patients' sera showed the haemolysin on the occasion of the first test[2].

As there had been a failure to demonstrate the presence of the haemolysin in several aborter series from the United States and Canada[2], and Budapest[1] it was decided to examine the Bantu and Indian populations of Natal.

MATERIAL

Specimens of clotted blood were obtained from Bantu and Indian women who were patients in the gynaecological ward of King Edward VIII Hospital, Durban. It was established that they were pregnant at the time of taking the specimen and were in danger of aborting.

The material was subdivided into two groups:

1. Habitual aborters, having experienced 3 or more abortions, repeated or "recurrent" abortions, or 3 or more miscarriages.
2. Likely habitual aborters having experienced 1 or 2 abortions, having a history of abortions, stillbirths and neonatal deaths, or whose obstetric history was considered by the physician to be "poor".

One specimen only was received from each woman.

METHOD

The serum was separated from each specimen within 12 hours of taking.

Two test tubes were prepared; one volume of serum and one volume of a 2% saline suspension of fresh group O cells was added to one, and one volume of serum and one volume of saline to the other. Both tubes were incubated at 37°C for one hour and then examined by visual inspection for haemolysis. The test tube without a cell suspension acted as a negative control.

RESULTS

There were 21 Bantu and 17 Indian samples tested in group 1, and 107 Bantu and 53 Indian samples in group 2.

No evidence of haemolysis was detected in any of the sera from either group.

DISCUSSION

As it had been anticipated that 39% of the specimens tested would have been positive for haemolysis[2], approximately 8 Bantu and 7 Indians in group 1 and 42 Bantu and 21 Indians in group 2 were expected.

As none at all were found in either group the presence of the anti-Tj^a-like haemolysin in Bantu and Indians in Natal seems unlikely and the

view is favoured that there are local environmental factors present in Perth, Western Australia.

SUMMARY

198 samples of serum from Bantu and Indian pregnant aborters in Natal were examined for the presence of anti-Tj^a-like haemolysins, but these were not found.

The view that there may be local environmental factors in Perth, Western Australia, is supported.

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Study 8

The P_1H antibody

Pages 204-221 in Ph.D. thesis, University of Natal, 1980.

Introduction

In 1967, and antibody of unknown specificity which reacted weakly in saline tests at 20°C with a few cell samples from group O Negroes (Zulus) was detected in the serum of a group B Indian blood donor. Microscopically, the agglutination was similar in type to that observed when (among other antibodies) anti-P_1, human anti-H and anti-I reagents agglutinate red cells. The reaction might even be described as "mixed field" in type, for while some of the cells were agglutinated more strongly than others, a minority appeared unagglutinated. A further example of this antibody was discovered in the serum of a White donor, and another in the serum of a second Indian donor, in 1968, and the opinion of Dr Giles in London was sought. Dr Giles suggested that the antibody was a type of anti-P_1 but made no other comments.

Results

Titrations

Since all the cell samples positive so far with the anti-P_1-like antibody had been from Zulus, and Zulu cells were known to have strong H antigen (Brain, 1968), in 1978 the H and P_1 antigen strengths of some newly-detected P_1-like positive cell samples and negative control cells of the same ABO group were estimated in careful parallel titrations using measured volumes of Ulex anti-H and several anti-P_1 reagents. The cell samples for the titrations were selected for being of the same age, and 5% suspensions of them were prepared in 1 mℓ volumes of the red cell suspending fluid of Burgess and Vos (1971) using measured volumes of washed, packed cells (matched cell suspensions). The anti-P_1 reagents had been standard-ised for use by different techniques. The results showed that the 15 P_1-like positive group O samples tested all gave unusually high H and high P_1 antigen titre results and scores with these reagents, while among the 10 P_1-like negative control group O samples, some gave similar high H but lower P_1, others lower H but similar high P_1 and still others lower H and lower P_1 titres and scores with them. A similar pattern of reactions was observed when two P_1-like positive and one negative group B samples were tested in the same way (Table 16.1.1). Group A and group AB cells could not be tested as the three donors of the anti-P_1-like antibodies were all group B.

Family studies

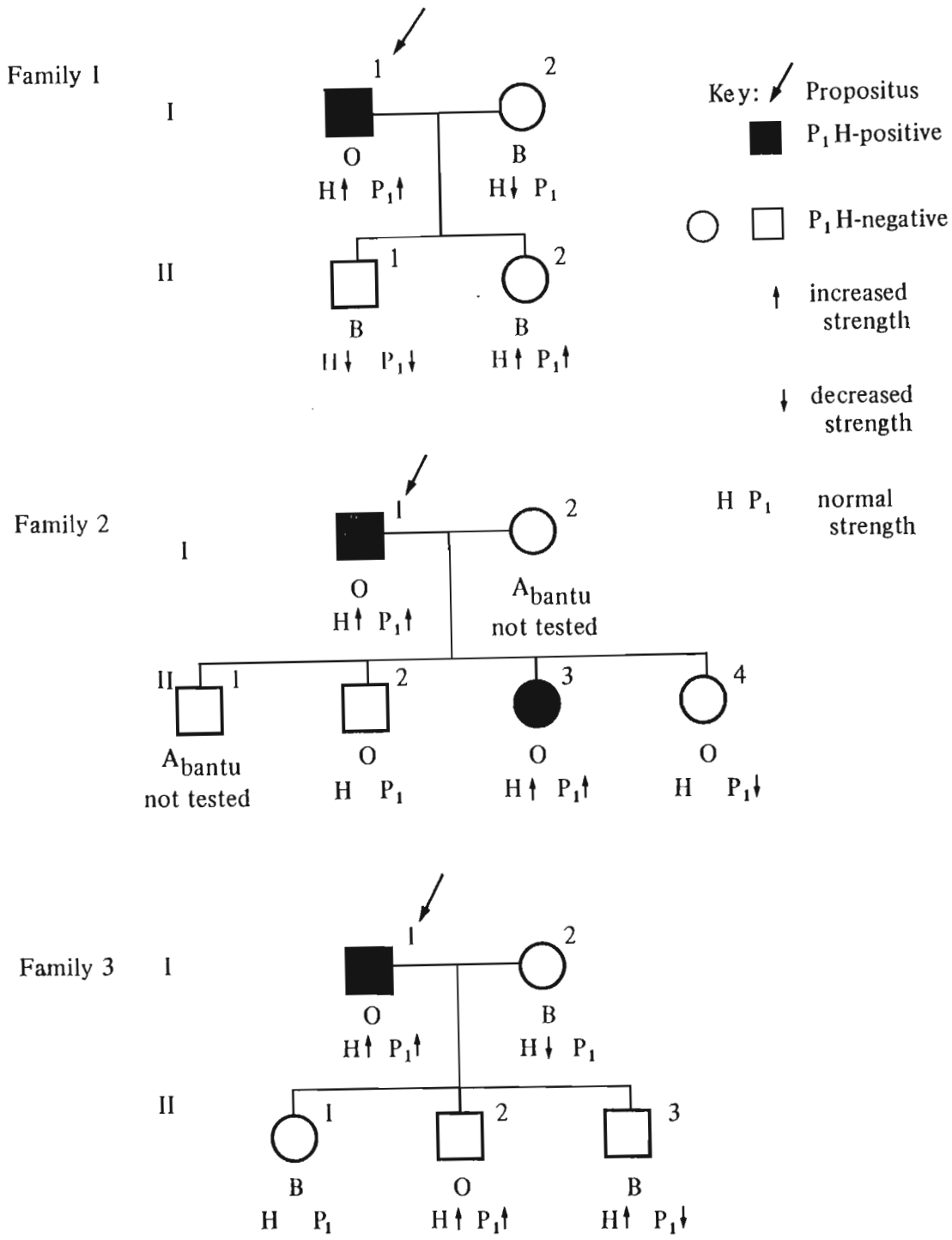
Three family studies (Figure 16.1) were made in 1978 with the much valued help of the Eastern Province Blood Transfusion Service in Port Elizabeth. The propositi (adult Xhosa Negro men who were all blood donors) were all found and members of their immediate families tested by this Service using a sample of the anti-P_1-like antibody sent to Port Elizabeth by myself for this purpose. On being received in Durban subsequently, the cells of the family samples were tested for their H and P_1 antigen strength by the technique described, and the concurrent exceptionally high titres and scores obtained with the cells of the three propositi using both the anti-H and the anti-P_1 reagents were noted. The cells of a daughter (group O) of one propositus, which were also agglutinated by the anti-P_1-like antibody, as expected were agglutinated both by the anti-H and the anti-P_1 reagents almost as strongly as those of her father. However, the cells of all the P_1-like negative family members tested were seen to react less strongly with both the anti-H and anti-P_1 reagents, or strongly with one of these reagents, but in no instance as strongly with both of these

TABLE 16.1.1

Titrations showing that anti-P_1 – like positive cells have strongly-expressed H and P_1 antigens

Sample No.	ABO group	P_1-like type	Anti-H dilutions 1	2	4	8	16	32	64	128	256	512	Score	P_1 type	Anti-P_1 dilutions 1	2	4	8	16	32	64	128	Score
1	O	+	4	4	4	4	4	3	2	(3)	–	–	70	+	4	3	2	1	1	(2)	–	–	39
2	O	+	4	4	4	4	4	3	2	(2)	–	–	69	+	4	3	3	2	1	(2)	(±)	–	44
3	O	+	4	4	4	4	3	2	1	(1)	–	–	63	+	3	3	2	1	(2)	(±)	–	–	34
4	O	+	4	4	4	4	4	3	2	1	(±)	–	73	+	4	4	3	3	1	(1)	–	–	45
5	O	+	4	4	4	4	4	4	3	1	(±)	–	75	+	4	4	3	2	1	(2)	–	–	44
6	O	+	4	4	4	4	4	4	4	2	(3)	–	80	+	3	3	2	1	1	(3)	–	–	40
7	O	+	4	4	4	4	4	3	1	(2)	–	–	66	+	4	4	3	3	2	1	–	–	53
8	O	+	4	4	4	4	4	3	2	(1)	–	–	68	+	4	4	3	2	1	(2)	–	–	44
9	O	–	4	4	4	2	1	(1)	–	–	–	–	43	+	4	3	3	1	(2)	–	–	–	36
10	O	–	4	4	4	3	2	1	(1)	–	–	–	53	+	2	1	(2)	(1)	–	–	–	–	14
11	O	–	4	4	4	2	(3)	–	–	–	–	–	40	+	1	(3)	(2)	(1)	–	–	–	–	8
12	O	–	4	4	4	4	4	2	(3)	–	–	–	60	+	3	3	2	1	(1)	–	–	–	33
13	O	–	4	4	4	3	3	1	(1)	–	–	–	55	+	3	2	1	1	(1)	–	–	–	28
14	O	–	4	4	4	3	3	1	(1)	–	–	–	55	+	4	3	2	1	(3)	(2)	–	–	36
15	B	+	3	2	1	(3)	(2)	(1)	–	–	–	–	26	+	4	3	2	1	(3)	(1)	–	–	35
16	B	+	3	3	2	1	1	(2)	–	–	–	–	39	+	3	2	1	(3)	(1)	–	–	–	25
17	B	–	4	3	2	2	1	(3)	–	–	–	–	43	+	1	1	(3)	(1)	–	–	–	–	12
Control	O	–	4	4	3	3	2	1	–	–	–	–	53	+	4	4	3	2	1	–	–	–	43
Control	B	–	3	2	1	(2)	–	–	–	–	–	–	24	+	2	1	(3)	(2)	–	–	–	–	16
Control	Cord O	–	2	1	1	(±)	–	–	–	–	–	–	18	+	–	–	–	–	–	–	–	–	0

Figure 16.1
Inheritance of P_1-like (P_1H) factor, strong H and strong P_1 antigens in three Xhosa Negro families



Note: In Family 3, II-2 had lower H and P_1 red cell antigen strength than I-1

In Family 1, II-2 had relatively high H and P_1 red cell antigen strength but not enough for his cells to be P_1 H-positive

reagents concurrently as the cells of the propositus in their families.

Inhibition tests

Inhibition tests using cyst fluid containing P_1 substances, saliva containing (1) H and Lewis substances from a group O secretor, (2) Le^a substance from a group O non-secretor, (3) H substance only from a group Le(a-b-) secretor, and Guinea-pig urine containing Sd^a substance showed that the anti-P_1-like antibody was inhibited only by the cyst fluid. Further tests, in which the cyst fluid was used both unbuffered and buffered to pH 7,0, also confirmed that this substance did not inhibit anti-P_1 antibodies non-specifically through being over-alkaline (Marsh and Oyen, 1978).

Frequencies

The frequencies of the curious "new" P_1H-positive phenotype in random group O Indians, Zulu Blacks and Whites in Natal are presented below. The highest P_1H-positive frequency was noted to occur in the Zulus, possibly reflecting their known high mean H red cell antigen strength (Brain, 1968). The χ^2 tests for significance in the frequencies between the three races were as follows:

Populations	χ^2	Probability
Natal Indians versus Natal Whites	2,8	not significant
Natal Indians versus Natal Zulus	72,0	<0,001
Natal Whites versus Natal Zulus	93,8	<0,001

Discussion

In selecting a suitable name for the anti-P_1-like antibody, the term anti-P_1H was chosen to indicate that it appeared to be recognising a product of strong P_1 and strong H antigen produced when these antigens were present on the same cell simultaneously but not when either strong P_1 or strong H antigen was present on them separately. As with the anti-IH of Rosenfield, Schroeder, Ballard, van der Hart, Moes and van Loghem (1964), the inclusion of the letter H was not meant to suggest that the antibody was inhibited by H substance. Moreover, no evidence suggesting that the anti-P_1H antibody was reacting in the same manner as the anti-IP_1 of Issitt, Tegoli, Jackson, Sanders and Allen (1968) was found, and no studies with examples of pure anti-I (as distinct from anti-IH), which suggested that the cells of some Natal Negroes had stronger than usual I antigen, were known to me.

The anti-P_1H antibody was thought possibly to be reacting with an antigenic structure or structures formed by steric interaction between strong P_1 and strong H determinants on the red cell membrane. This was not unexpected as the biochemists have shown that the antigens of the ABO and P_1 systems are closely associated at the molecular level. It appears that a precursor, lactosyl ceramide, may be converted through ceramide trihexoside (P^K) to globoside (P) or through the sequential action of N-acetylglucosamine and galactose to paragloboside (lacto-N-neotetraosyl ceramide). The paragloboside is then converted either to a type 2 H structure (and so to A and B structures), to a P_1 determinant by the addition of an alpha-galactosyl residue, or to sialoparagloboside by the addition of sialic acid (Watkins, 1980, p 109). The biochemists' views on the specificity of the anti-P_1H antibody will be most welcome. The P_1H family studies suggested that one but not both O genes in the propositi encoded increased H antigen expression. Since no family member was P_2, however, it was not possible to assess the effect of the inheritance of the $\underline{P}^1$ gene. Further work therefore seems essential.

The low P_1H-positive frequencies, which failed to distinguish the Natal Indians from the Natal Whites but distinguished both markedly from the Natal Blacks, agreed with the view that the Natal Indians had little Black admixture.

Summary

The P_1H-positive frequencies in the Natal Indians and the Natal Whites were similar, but they differed significantly from the P_1H-positive frequency in the Natal Blacks.

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CHAPTER IV

THE Rh SYSTEM

IV.1 PAPERS

Haemolytic disease of the newborn. A report from the Natal Rhesus Unit
Paper 1 by Wallace, Edge, Mann and Moores

The serological diagnosis of haemolytic disease of the newborn
Paper 7 by Moores

The incidence and severity of jaundice in the newborn in Salisbury, Southern Rhodesia
Paper 8 by Hunt, Moores and Plampin

The third example of anti-hr^s
Paper 9 by Grobbelaar and Moores

Serology and genetics of the red blood cell factor Rh34
Paper 53 by Moores and Smart

STEM, a new low frequency antigen linked to Rh and associated with the red blood cell phenotypes hr^s- (Rh:-18,-19) and hr^B- (Rh:-31,-34)
Paper 56 by Marais, Moores, Smart and Martell

Rh33 antigen in two of three West German siblings with D+C+c+E-e+ red cells
Paper 55 by Moores, Smart, Sternberger and Schneider

The Rh haplotype *D*-- identified in five Cape Coloured families
Paper 52 by Mulvihal and Moores

D-- and *Dc*- gene complexes in the Coloureds and Blacks of Natal and the Eastern Cape and blood group phenotype and gene frequency studies in the Natal Coloured population
Paper 54 by Moores, Vaaja and Smart

Rh_{null} red cells and pregnancy
Paper 37 by Gibbs and Moores

A case of hereditary ovalocytosis
Paper 10 by Moores and Buck

IV.2 INTRODUCTION

Landsteiner and Wiener discovered the Rh groups in 1940. The sera of Guineapigs and rabbits, lately immunised by them with red blood cells from Rhesus monkeys, was found to contain antibodies which agglutinated the red cells of 85% of people selected at random in New York. The previous year, Levine and

Stetson had reported demonstrating an antibody in the serum of a mother who suffered a severe transfusion reaction when her second child was born. They suggested that the antibody had been stimulated by an immunising property in the fetus, that the fetus had inherited from its father but was absent in its mother. In 1940, Wiener and Peters realised that antibodies similar to the Guineapig and rabbit anti-Rhesus were also present in some human sera and could cause haemolytic transfusion reactions. Levine, Katzin, Burnham and Vogel showed in 1941 that erythroblastosis fetalis, or haemolytic disease of the newborn, was due to Rh group incompatibility between mother and child.

Anti-hr^S, which has a e-like specificity, was first described in 1960 by Shapiro. The red cells of most people were e+hr^S+, but those of a few South African Blacks were e+hr^S-. The Rh gene complexes associated with the hr^S- phenotype were $\hat{R}^o$, $\hat{R}^{ou}$ and $\hat{R}^2$. Anti-hr^B, another e-like antibody, was described in 1972 by Shapiro, le Roux and Brink. The red cells of most people were e+hr^B+, but those of a few other South African Blacks were e+hr^B-. The Rh gene complexes associated with the hr^B- phenotype were $\dot{R}^o$, $\dot{R}^{ou}$, $\dot{R}^2$, $\dot{r}'^n$ and $\dot{r}$.

The rare antigen, Rh33, was described in 1971 by Giles, Crossland, Haggas and Longster. The German propositus, in whom the gene responsible was apparently homozygous, had normal c, variant D and depressed e antigens on his red cells. The English propositus was initially thought to be Rh negative. The first example of red cells with the rare D--, or "D-deletion", phenotype that behaved as though some of its antigens were either missing or inhibited, was discovered in 1950 by Race, Sanger and Selwyn. People with homozygous *D--/D--* genes had enhanced expression of D and G but no evidence of C, c, E or e. The *Dc-* gene, encoding enhanced D, normal c and depressed or unusual e, was described in 1960 by Tate, Cunningham, McDade, Tippett and Sanger. Rh_{null} red cells, with no demonstrable D, G, C, c, E or e antigens, were first found in 1961 in an Australian Aboriginal woman by Vos, Vos, Kirk and Sanger.

Linkage between genes at blood group and non-blood group loci was first demonstrated in 1953 by Chalmers and Lawler. The loci were for Rh and for oval-shaped red cells, or elliptocytes. Hereditary elliptocytosis was reported by Dresbach as early as 1904 [Dacie, 1960]; in 1941, Wyandt, Bancroft and Winship estimated that this condition occurred in approximately 1 in 2500 random people in the United States.

IV.3 COMMENTARY

IV.3.1 Haemolytic disease of the newborn

IV.3.1.1 Attainments in Durban, 1949-1955

During the author's first period in Durban, a "Rhesus Unit" was established, with a pediatrician in charge and herself in the laboratory. The intention was to provide a centre where the treatment for haemolytic disease of the newborn would be standardised. The project received the overwhelming support of hospital and independent doctors throughout the city. Paper 1 described the results of 112 pregnancies and the technique used in exchange transfusion. Guidelines for future management were also provided. Twelve pregnancies terminated in hydrops fetalis, stillbirths or abortions. Twenty babies were Rh negative. The remaining 80 babies were all affected to varying degrees. Routine exchange transfusion was not practiced, but the maternal antibody titre, aspects of the obstetric history, the baby's birth weight and spleen size and the results of the direct antiglobulin and serum bilirubin tests, were all carefully considered. The findings showed that the 80 cases were subdivisable into two groups: (1) those requiring and (2) those not requiring immediate exchange transfusion. In both groups, some babies needed straight transfusions later because their haemoglobin fell rapidly during the neonatal period. The overall mortality rate was 10%.

IV.3.1.2 Attainments in Salisbury, 1957 to 1961

Paper 7 contained a detailed account of the history and serological diagnosis of haemolytic disease of the newborn due

to immune anti-A, immune anti-B and anti-D. Some information on the process of mitosis and meiosis was also included. The genes in the ABO and Rh systems were described, and family studies were shown in order to illustrate gene inheritance. The significance of the D^u factor, the general management of Rh pregnancies at that time and the technique of exchange transfusion were all discussed. Paper 7 was read at a meeting of the Central African Society of Medical Laboratory Technologists, and it was subsequently published in full in the society's journal.

The studies described in paper 8 were intended to determine why so many full term White babies in Salisbury with normal birth weights and no evidence of Rh antibodies became jaundiced soon after birth and required exchange transfusion to prevent kernicterus. The incidence of jaundice due to immune anti-A or anti-B was within normal limits. The most likely explanation, although not proved, was dehydration in the nursery environment. A warning was given that the practice of dismissing jaundice in the newborn as merely physiological was potentially hazardous, without first having excluded as the cause haemolytic disease of the newborn due to immune anti-A and/or anti-B.

IV.3.2 Rh antigens and antibodies

IV.3.2.1 Attainments in Durban, 1961 to 1991

IV.3.2.1.1 Anti-hr^s

A Black woman was identified in Durban with R_2r (DcE/dce) red cells and anti-hr^s in her plasma, one year after Shapiro had described this antibody. Paper 9 contained an account of the results. The woman, Mrs Sakwe, had given birth in 1959 to an infant who was thought but not proved to have suffered mildly from haemolytic disease of the newborn. At the time, the causative antibody was not identified. In 1961, her stored serum was re-investigated and her antibody found to have apparent anti-e specificity. This was strange, for the earlier records showed that her red cells were e+. By co-incidence, a few weeks later a fresh specimen of blood was received for

antenatal tests. Mrs Sakwe's DcE/dce phenotype and e-like antibodies were confirmed. Dr Shapiro kindly identified her phenotype as $Rh_2\hat{R}h_o$ and her antibody as anti-hr^s. He also said that a weak result with papainised R_2R_2 red cells, which he said was due to anti-Hr. The sera and red cells of Mrs Sakwe and Dr Shapiro's proposita, Mrs Shabalala, were mutually compatible.

IV.3.2.1.2 Anti-Rh34 and anti-hr^B

Following 26 years of investigations with 36 sera containing e-like antibodies, the reactions with all of which resembled anti-hr^B, a breakthrough was made. The antibodies had anti-Rh34 specificity [paper 53]! Previously, this specificity had been described as a mixture of anti-hr^B and anti-Hr, which was compatible only with Rh "deleted" and Rh_{null} red cells. The anti-Hr was absorbed with R_2R_2 red cells, leaving "pure" anti-hr^B in the serum. Moreover, anti-hr^B was a possible component of many anti-e reagents. The authors' found instead that the red cells of numbers of South African Blacks with ordinary Rh phenotypes were compatible with the unabsorbed Rh34 antibodies. Four haplotypes that encoded Rh34 antigen were identified (they are indicated here by *). The haplotypes were $*r'^s$, $*R^o$, $*R^{ou}$ and $*R^{od}$. Furthermore, anti-Rh34 was a useful reagent for disputed paternity studies. When titrated, it also recognised *RH 34* dosage. The frequency of Rh:-34 phenotypes in Natal Black and Coloured blood donors with Rh D+ red cells was 0,27%, and in those with Rh D-C+ red cells 4,08%.

IV.3.2.1.3 The STEM antigen and antibody

Studies with the antibodies of a Coloured woman living in Cape Town showed that the low frequency antigen on her husband's red cells was linked to Rh and closely associated with the e-like phenotypes hr^s- (Rh:-19) and hr^B- (Rh:-31,-34). The antigen was given the name STEM, the proposita's surname [paper 56]. It was inherited as a dominant Mendelian character, and subdivided both e-like phenotypes into two kinds, STEM+ and STEM-. The Rh genes which encoded STEM were $\hat{R}^o$, $\hat{R}^{ou}$ and $\dot{R}^o$. The strength of STEM antigen varied on the red cells of different individuals,

and the different strengths were inherited. The frequency of STEM+ red cells in Blacks and Coloureds who had not been typed for hr^s or Rh34 varied from 3,1% to 5,9% in different regions. The gene frequency was from 0,016 to 0,030 respectively. The proposita's anti-STEM was an IgG antibody that reacted best by enzyme techniques; its titre was 16-32. Further examples have since been identified. As approximately two of every three people with hr^s- (and one of every three people with Rh:-34) red cells was STEM+; this antigen was a useful marker when screen-testing to find hr^s- blood.

IV.3.2.1.4 Rh33 antigen

Two of three siblings with R_1r phenotypes in a family living in Hagen, Germany, were found to have rare Rh33 red cells [paper 55]. The proposita's Rh33 was stronger than that of her sibling. Her C, e, f, Rh19 and Rh34 antigens were also expressed weakly, and no Rh17 antigen was demonstrable. The findings suggested that the proposita had an R^{oHar} haplotype and a new haplotype, which we provisonally named R^{1Lisa}. The new haplotype encoded Rh33, normal strength D, weak C, weak or non-demonstrable e, Rh19, Rh34 and no Rh17 antigens. Her Rh:33 sibling evidently had R^1 and R^{1Har}, and her Rh:-33 sibling R^1 and r, haplotypes.

IV.3.2.1.5 *D*-- and *Dc*-

During disputed paternity testing, twenty-one persons who were apparently heterozygous for the rare Rh haplotype *D*-- were identified [paper 52]. This haplotype had not been recorded before from South Africa. The red cells gave 1+ to 3+ reactions with incomplete anti-D reagents which were known to detect enhanced D antigen in saline tests. In titrations with anti-C, -c, -E, -e, -Rh18 and -Rh34 (where relevant), their red cells also gave lower titres and scores than those of their family members and of other persons with similar Rh phenotypes who had ordinary *RH* genes. The *D*-- heterozygotes were all members of four Cape non-Malay and one Cape Malay Coloured families. One member of one Cape non-Malay family had

r'^{s}R$_{o}$,Rh:-34 and one member of another R$_{o}$,Rh:-18 red cells. Their genotypes were evidently *D--/dCCe^{s}*(R^{-34}) and *D--/R^{o-18}*, respectively. One member of the Cape Malay family had R$_{o}$,Rh:-34 red cells; his genotype was evidently *D--/R^{o-34}*.

The haplotype *D--* was again identified in a Coloured family from the Eastern Cape. In a Natal Coloured family, an unusual haplotype encoding weak e and very weak or no f antigens was also recorded (paper 54). The latter almost certainly belonged to the heterogenous collection known as *Dc-*, but no examples had been recorded before from South Africa. The Natal haplotype was found when Rh phenotypes in the family members expected to be f+, instead were f+^{w} or f-, and when Rh phenotypes in family members expected to be f-, instead were f+^{w}. Studies in Natal Coloured blood donors showed that the *DCe/Dc-* frequency was 6,9%, and *DcE/Dc-* frequency 2,6%. The *Dc-* haplotype appeared to be a characteristic of the Black race. The phenotype and gene frequency studies in Natal Coloured blood donors recorded in paper 54 are included in Chapter XII.

IV.3.2.1.6 Rh$_{null}$

A White woman living near Cape Town was found to have red cells with no detectable Rh antigens [paper 37]. Her red cells were also LW-, Ms U-, I+ and i+^{w}. She was pregnant, and her plasma contained anti-Rh29; the initial titre was 4. A family study showed that the woman's Rh phenotype was the "regulator" type, which is the result of the inheritance of homozygous *X°r* genes. As no suitable blood donors were available and the woman was in excellent health, two units of blood were withdrawn from her, one at 20 and the other at 27 weeks, and stored in liquid nitrogen. The blood loss was well-tolerated. Amniocentesis was performed as a precaution at 37+ weeks, and the optical density reading at 450 nm alarmingly discovered to be in the upper middle zone of Liley's chart. Consequently, delivery was effected by caesarian section at 38 weeks. The infant proved to be only mildly affected, however, and required no blood transfusion. As no evidence was seen in the mother of the

normocytic, normochromic anaemia reported in other cases had been seen, we concluded that Rh_{null} red cells need not necessarily be a cause for concern. We were satisfied that the pregnancy had been managed correctly and that the infant had not required the early delivery.

IV.3.2.1.7 *r* (*dce*) and oval red cells

Oval-shaped red cells (or elliptocytes) were identified in 1963 in a White woman when her recent blood donation was cross-matched for a hospital patient [paper 10]. A family study was made and the gene responsible seen to have segregated with an *r* (*dce*) gene complex in the eleven affected members. Decreased red cell survival was demonstrated but was evidently being compensated-for adequately.

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HAEMOLYTIC DISEASE OF THE NEWBORN

A REPORT FROM THE NATAL RHESUS UNIT

H. L. WALLACE, M.D., F.R.C.P.

W. E. B. EDGE, M.B., M.R.C.P.

N. M. MANN, M.D., M.R.C.P., D.C.H.

and

PHYLLIS MOORES, B.Sc.

Addington Children's Hospital, Durban

In 1951, the Director of the Durban Blood Transfusion Service, Dr. J. C. Thomas, put forward the suggestion that a 'Rhesus Unit' should be established at Addington Hospital, Durban, to serve not only Durban but the whole Province of Natal, and to which all Rhesus-immunized women in the province might be referred for their confinements. The advantages of such a scheme seemed obvious in that adequate facilities, both laboratory and clinical, are much more readily available at a large specially-equipped centre than in outlying country districts or even in private nursing homes. The concept of centralization of these difficult cases was wholeheartedly endorsed by the obstetrical and paediatric staffs of the hospital and, accordingly, an explanatory circular was sent to all medical practitioners in the province inviting their cooperation. In the Durban area the response to this appeal has been excellent and it is probably a true statement that in the past 4 years the vast majority of Rhesus-immunized women in the area have been delivered at Addington Hospital. The use made of the unit by the country districts is more difficult to assess, since the number of cases diagnosed in these districts is problematical. Despite the relatively small number of country cases, however, it is encouraging to note that these have come from all parts of the province, and the majority have done well.

At Addington Hospital there is a close liaison between the obstetrical and paediatric departments, and all infants born there come under the care of the paediatric staff, who are thus responsible for deciding on and carrying out the treatment of the newborn infant with haemolytic disease.

The procedure adopted by the Rhesus unit has been standardized as far as possible. The prospective mother's blood group is determined during early pregnancy. If she is Rh-negative antibody tests are carried out and repeated at 24, 32 and 36 weeks if practicable. Should Rhesus antibodies develop admission to hospital is advised in the 38th week.

Immediately after delivery clotted and unclotted samples of cord blood are sent to the special Rhesus laboratory, where the following tests are carried out:

1. The direct Coombs anti-human-globulin test
2. Haemoglobin estimation
3. Blood and Rhesus grouping
4. Serum bilirubin estimation
5. Normoblast count.

Whilst the above tests are being carried out in the laboratory the newborn infant is subjected to a careful clinical examination, during which particular attention is paid to the weight, the state of the general health, the presence or absence of jaundice, and the size of the liver and spleen. Details of the mother's obstetrical history are also carefully assessed. Essential laboratory and clinical investigations are usually complete within an hour of birth and it is possible, therefore, to institute the required treatment with the minimum of delay.

Town and Country Cases

The series under consideration comprises a total of 112 cases admitted to Addington Hospital during the 4 years 1951-55. Of these, 84 were admitted from the Durban area, the remaining 28 being referred from various country towns throughout the province of Natal.

Stillbirths etc.

The total of 112 cases includes 12 pregnancies which terminated in hydrops foetalis, stillbirth or abortion. For the purpose of this paper these regrettable cases will be excluded from our analysis, which is concerned primarily with the mangement of the affected live-born infant. Suffice it to observe that, where there is a history of one or more stillbirths and where the husband is homozygous, early induction of labour or Caesarean section might possibly increase the chances of procuring a live infant. Such a policy was not generally adopted in this series but will be carefully considered in future cases, bearing in mind the apparent advantages of early induction in selected cases as reported by Kelsall and Vos.[1]

Live-born Infants

During the 4 years 100 live-born infants were born of Rh-sensitized mothers. Although antibodies were detected in the maternal blood, 20 of these infants proved to be Rh-negative and, being unaffected, did not require any treatment. The remaining 80 Rh-positive infants were all affected to a greater or less degree and their treatment will be discussed in some detail with

particular reference to the indications for exchange transfusion.

The total number of cases presenting in the 4 years may be tabulated as follows:

Stillbirths and abortions ..	12 cases
Infant Rh. —, Coombs test — ..	20 cases
Infant Rh. +, Coombs test + ..	80 cases
Total	112 cases

Indications for Exchange Transfusion

In the treatment of haemolytic disease of the newborn the merits of exchange transfusion have been proved by a number of investigators.[2,4] Compared with other forms of treatment not only is the survival rate higher, irrespective of the birth weight or of the severity of the disease,[4] but the incidence of kernicterus is reduced by at least two-thirds. Impressed by these undoubted virtues, some units still perform exchange transfusion in all cases where the cord blood gives a positive Coombs anti-human-globulin test. Not only is this a waste of precious Rh-negative blood, but it exposes unnecessarily a considerable proportion of infants (estimated at 30-40% of those affected) to the risks of the operation, viz. shock, air-embolism, infection, incompatible transfusion, portal-vein thrombosis and perforation. Apart from the first we have as yet encountered none of these mishaps, but they are too real to be ignored.

In an attempt to define the indications for exchange transfusion Mollison and Cutbush[5,6] first drew attention to the value of the cord haemoglobin as an index of severity, taking 14·8 g.% as the critical value. Subsequent studies[3,4] on their untreated infants revealed an incidence of kernicterus of 7% in the mature and 14% in the immature groups, and these workers have, therefore, revised their criteria and now regard as indications for immediate exchange tranfusion (in the presence of a positive Coombs test):[7]

1. A birth weight of 6 lb. or less, or an infant born 3 weeks or more before the expected date of delivery (irrespective of other findings)
2. A history of the mother having previously given birth to an affected infant (irrespective of other findings)
3. A cord haemoglobin below 15·5 g.%

They point out that in cases where the cord haemoglobin lies between 15·5 and 17·5 g.% there is still a possibility of kernicterus occurring, and in such cases the onset of jaundice within the first 24 hours should be an indication for treatment. Walker and Neligan,[8] discussing this same problem, regard the cord bilirubin level as being of considerable help, and in cases where the cord haemoglobin lies between 14·8 and 17·7 g.% a bilirubin value of 2·8 mg.% or above is taken as an indication for exchange tranfusion. Other factors which are recognised as being of some assistance in assessing border-line cases are: The degree of positivity of the Coombs test, the maternal anti-Rh titre and the nucleated red-cell count on the cord blood. We would also include splenic and hepatic enlargement.

In the Durban unit we have not favoured the practice of routine exchange transfusion in all affected infants but have attempted to differentiate between those requiring immediate treatment and those which could safely be left untreated. Sometimes the selection of cases for treatment presents little difficulty, as in the severely affected infant whose precarious state is apparent from the moment of birth. More often the decision entails the careful appraisal of a variety of factors. Whilst following fairly closely the indications for exchange transfusion suggested by other units, our standards may have been less rigid in that the decision for or against treatment has not always depended on pre-elected laboratory findings. For example, although the cord haemoglobin level has proved a most valuable guide in all cases, we have not necessarily been bound by any arbitrary figure but rather have considered the haemoglobin level in relation to the many other relevant factors. A further point is that the ultimate decision regarding treatment in each case has rested on one individual, a policy which, we believe, makes for uniformity and consistancy of approach.

It is now proposed to discuss the foregoing guiding principles in the treatment of haemolytic disease of the newborn in relation to this series of cases. To simplify description the latter have been divided into 2 main groups, viz.: *Group I*—immediate exchange transfusion performed (50 cases). *Group II*—no immediate treatment given (30 cases).

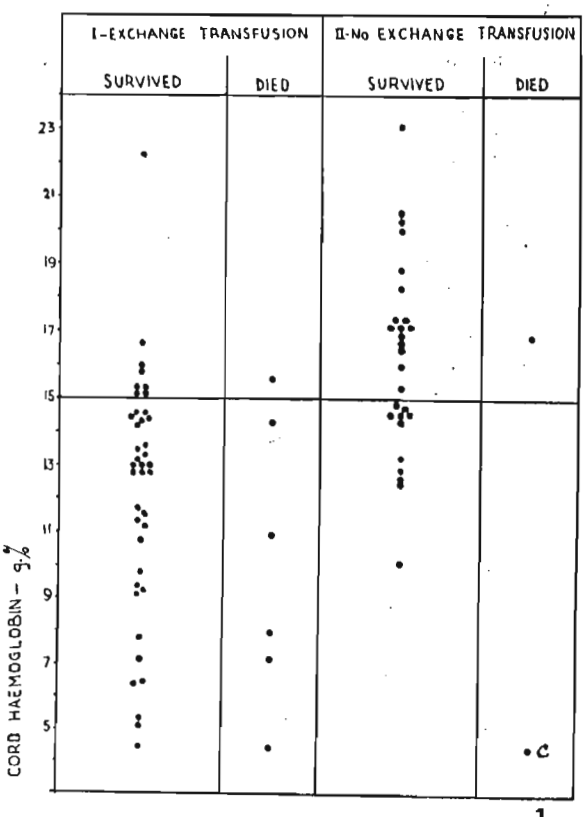

Fig. 1. Cord haemoglobin levels. C=due to anti-c, undetected antenatally.

Birth Weight. There were 14 infants whose weight was less than 6 lb. at birth. Of these, 12 were given immediate exchange transfusion and 2 were left untreated. One of the treated cases with a birth weight of only 4 lb. 14 oz. died, and the remainder made a good recovery. We do not feel convinced, provided the other factors are satisfactory, that all infants weighing 6 lb. or less at birth require immediate treatment.

Mother's Obstetrical History. As already mentioned, the previous obstetric history of the mother must influence the decision whether her infant should receive immediate treatment. In this series 16 mothers gave a history of having had one previous infant affected and in 4 of these the new infants were left untreated and made an uneventful recovery. A further 11 mothers had had more than one affected infant and in all these cases immediate exchange transfusion was carried out. It is suggested that a history of only one previous infant affected is not an absolute indication for immediate treatment, but that where there have been 2 or more infants affected exchange transfusion should usually be carried out.

Cord Haemoglobin. Fig. 1 shows that the majority of cases having exchange transfusion had cord haemoglobin levels below 15 g.%. Those above this level had other indications for treatment. It will be noted that 11 cases with haemoglobin levels below 15 g.% were left untreated and made a good recovery, and it is probable that a considerably higher proportion of cases in this category could be spared unnecessary operation by careful assessment of other factors. It is of interest to note that one case which was left untreated and died of kernicterus had a cord haemoglobin of 16·9 g.%.

Serum Bilirubin. Fig. 2 illustrates the serum-bilirubin values of the cord blood which was estimated in the

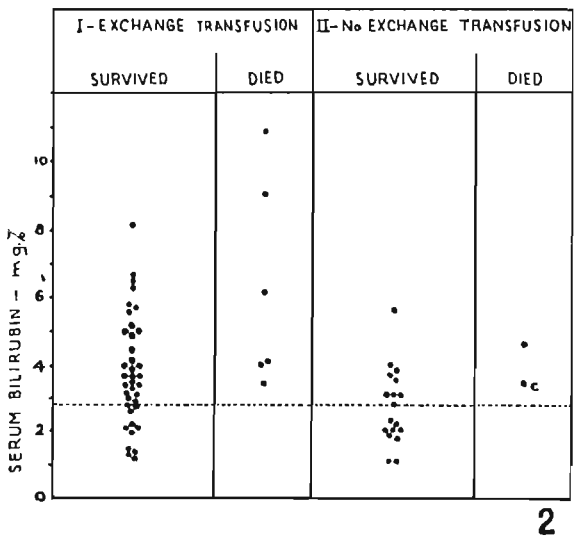

Fig. 2. Serum bilirubin levels. C=due to anti-c, undetected antenatally.

majority of cases. Though all the fatal cases had values above 3 mg.%, it will be noted that over 50% of the non-fatal cases also had values above this figure. A serum bilirubin of 4 mg.% or over would, however, appear to have some prognostic importance, since 28% of our cases in this category proved fatal.

The association between very high serum-bilirubin levels and kernicterus is well-established,[9] and it would therefore appear advisable to perform exchange transfusion and, if necessary, to repeat this procedure whenever the serum bilirubin rises to 20 mg.%, and perhaps less in premature infants. We have had occasion to repeat the exchange transfusion in one instance, with a successful outcome.

Degree of Positivity of the Coombs Test. This has been estimated in 4 degrees (in nearly all cases by the same technician), and eð udistribution in the 2 groups is shown in the followith table:

Coombs Test	*Group I*		*Group II*	
	Successful	*Fatal*	*Successful*	*Fatal*
++++	26	5	9	1
+++	8	1	4	—
++	3	—	5	1
+	7	—	10	—

The association of a strongly positive Coombtest with the need for exchange transfusion, and particularly with the fatal cases, is evident. No further conclusions can be drawn from this small series, but it is felt that in doubtful cases a strongly positive Coombs test should swing the balance in favour of exchange transfusion.

The Maternal Antibody Titre. Weiner[10, 11] has for long fought rather a lone battle in defence of the maternal anti-Rh titre as an index of the severity of the disease in the infant, although Mollison[7] has recently observed that a titre of over 64 can be correlated with an increased risk of kernicterus. Our own figures lend considerable support to Weiner's view as will be seen from the table.

Maximum Maternal Anti-Rh Titre (Albumin)	*Group I*		*Group II*	
	Successful	*Fatal*	*Successful*	*Fatal*
8,192	2	1	1	—
4,096	6	—	2	—
2,048	8	1	2	1
1,024	4	1	2	—
512	5	1	2	—
256	6	1	4	—
128	8	1	2	—
64	1	—	7	1
32	1	—	1	—
16	1	—	4	—
8	1	—	—	—
4	1	—	—	—
2	—	—	—	—

It will be noted that 45 of the 50 cases requiring exchange transfusion had a maternal antibody titre of over 64, and all the fatal cases, with one exception, belonged to this group. In view of these findings we feel that in doubtful cases a maximum maternal antibody titre of more than 64 is a definite pointer in favour of exchange transfusion.

The Normoblast Count. This we have found to be a very variable and somewhat unreliable test, but a count of 10 or more per 100 white cells has usually been

associated with severe disease. Our results show that, of the cases requiring exchange transfusion, 93% had a normoblast count of 10 or more, whereas in those cases which were left untreated only 62% had a count in this range. From a practical point of view, however, we consider the normoblast count to be of little value.

Splenomegaly. The degree of splenomegaly, where it was accurately recorded, may be depicted as follows:

Splenomegaly	*Group I*		*Group II*	
	Successful	*Fatal*	*Successful*	*Fatal*
++	8	4	—	—
+	19	2	5	—
0	12	—	20	1

It will be noted that 33 of the cases in group I, or 73%, had splenomegaly, as compared with only 20% in group II. Moreover, in the latter group there were no cases which exhibited gross splenic enlargement. We are well aware of the difficulty in assessing splenic enlargement in the newborn, but experience leads us to believe that a readily palpable spleen is usually indicative of severe disease.

To summarize this section concerning the various factors which influence the decision whether or not immediate treatment of the affected infant is indicated, it is suggested that there is a considerable group of cases with cord haemoglobin levels below 15 g.%, in which fine judgement can lead to a decrease in the number of exchange transfusions performed. In such cases knowledge of the birth weight and of the mother's obstetric history is of prime importance, and valuable guidance is obtained from the serum-bilirubin level, the maternal antibody titre, the positivity of the Coombs test, and the size of the spleen.

TECHNIQUE OF EXCHANGE TRANSFUSION

In the majority of our cases exchange transfusion was carried out by way of the umbilical vein in accordance with the technique originally advocated by Diamond in 1947.[12] It must be recorded, however, that in 5 cases where exchange transfusion was considered advisable it was found impossible to establish a satisfactory flow from the umbilical vein. In 2 of these a polythene catheter of 0·5 mm. bore was passed into an umbilical artery and a successful exchange performed by this route. We suggest that this technique is well worth attempting when difficulty is encountered with the umbilical vein. Approximately 70-80 c.c. per lb. body-weight of group-O Rh-negative blood were given and the same amount withdrawn. Immediately before the operation the donor blood was concentrated by decanting about 15% of the plasma. Latterly, after every 100 c.c. exchanged, 1 c.c. of 10% calcium gluconate has been injected into the vein.[13] Warming of the blood was not a routine precaution but it is probably advisable. Marting *et al.*[14] quote Wheeler's interesting suggestion that the transfusion of cold blood may precipitate cardiac arrhythmias. Although in this series no obvious cardiac irregularities developed, 2 infants showed signs of collapse towards the end of the transfusion but quickly revived when warmed up in an incubator. In fact, one of the difficulties has been to maintain body heat during a somewhat lengthy procedure, and the giving of warmed blood might help to resolve this difficulty.

A repeat exchange transfusion was given on the second day in one case when the serum bilirubin rose to 21 mg.% and jaundice became intense. Since the umbilical vein was no longer patent the exchange was successfully carried out through the saphenous vein in the right thigh,[15] and the infant made an excellent recovery.

RESULTS

The results obtained in this series of 80 infants affected with haemolytic disease of the newborn may be described briefly as follows:

Group I. In this group, comprising 50 cases, immediate exchange transfusion was performed. Complete recovery occurred in 43 cases, or 86%. One further case survived but is suffering from the effects of kernicterus, and the remaining 6 cases died. Four of the cases in this group required subsequent direct transfusions.

Group II. In this group of 30 cases no immediate treatment was given, although 7 were given subsequent direct transfusions during the neonatal period, when the haemoglobin showed signs of falling rapidly. This was a precautionary measure and possibly was not necessary in all cases. In this group, 28 infants recovered completely and 2 died.

The total results are shown in the following table:

Group	*Cases*	*Deaths*	*Mortality %*	*Kernicterus (surviving)*
I. (exchange transfusion)	50	6	12·0	1
II. (no exchange)	30	2	6·6	—
Totals	80	8	10·0	1

The over-all mortality of 10% is reasonably satisfactory and compares favourably with results from many other centres. The mortality figures include one case where death may not have resulted from haemolytic disease since autopsy revealed gross congenital defect of the left kidney and ureter. Another fatal case was due to anti-c and was undetected before death (see below). These 2 somewhat doubtful cases have been included in the over-all mortality rate of 10%.

Short summaries of the fatal cases are shown at the end of this article; these reveal some interesting facts which may serve as a guide to reducing mortality in future cases.

For those babies who died after exchange transfusion perhaps little could have been done, but in the light of more recent knowledge they might have been given the benefit of a second or third exchange if and when the serum bilirubin rose to 20 mg.%,[9, 16, 17] even though signs of kernicterus had already appeared. Moreover, it is just possible that those deaths that occurred within 24 hours of transfusion (cases D and F) might have been associated with electrolytic disturbances,[18] though the blood used was not old. The injection of calcium gluconate now forms part of our routine.

There remain the two cases (G and H) in which the patients died without exchange transfusion. Case G was the first affected child, was not premature, and had a cord haemoglobin of 16·9 g.%. Jaundice was first noticed 36 hours after birth. On Mollison's criteria, therefore, there was no indication for exchange transfusion, but Walker would have been influenced in its favour by the serum bilirubin of 4·4 mg.%. In retrospect we consider that the very strongly positive Coombs test and the high maternal antibody titre of 2,048 were additional factors which should have influenced us in favour of performing exchange transfusion in this case.

Case H was due to anti-c and occurred in an Rh-positive (D) woman who had received a blood transfusion 6 years previously. We feel that death in this instance was inevitable, since the incompatibility was unsuspected and the infant died 20 minutes after birth. Nevertheless, this unfortunate case must surely underline the danger of indiscriminate blood-transfusion in female children and young adults, and illustrates the necessity of searching for unusual antibodies in Rh-positive women who give a history of previous blood-transfusion.

KERNICTERUS

It will be noted in the summaries of the 8 fatal cases that signs of kernicterus were evident before death or at autopsy in 4. The majority of the surviving cases have been followed up for at least 6 months and, so far as it has been possible to ascertain, only one of these exhibits signs attributable to kernicterus. This infant was quite severly affected at birth with a cord haemoglobin of 12·0g.% and serum bilirubin of 5·8 mg.%, although there was no obvious jaundice. The Coombs test was strongly positive. Immediate exchange transfusion was performed and the infant was discharged from hospital apparently vigorous and well. Subsequently convulsive attacks became frequent and there is now gross mental defect and blindness. A happy sequel for the mother of this tragic case has been the recent birth of a healthy unaffected infant.

FOLLOW-UP

We are fortunate in being able to follow-up these cases in the clinic at Addington Hospital for babies born in the maternity section. Weekly haemoglobin estimations are carried out in all cases of haemolytic disease, and it is thus possible to observe their progress during the first few months of life. The majority of infants in this series have been followed up for at least 6 months.

The usual haemoglobin pattern has been a fairly steady fall until the 6th or 7th week, followed by a spontaneous and sustained rise. Provided the infant is thriving satisfactorily, our practice has been to withhold blood transfusion unless the haemoglobin level falls below 6 g.% (40% Haldane).

SUMMARIES OF FATAL CASES

Case A

First child normal; second, third and fourth children died of congenital haemolytic disease; fifth child unaffected.

Sixth child. Caesarean section. B.W. 10 lb. Cord Hb. 14·2 g.%. Cord bilirubin 4·0 mg.%. Coombs ++++. Hepato-splenomegaly ++. E.T. 825/800 c.c. Well, but slight jaundice on the following day. Signs of kernicterus on 3rd day. Increasing jaundice and death on 4th day. P.M. refused.

Case B

First child normal. Second child jaundiced and died on 3rd day.

Third child. B.W. 7 lb. 1 oz. Cord Hb. 4·4 g.%. Cord bilirubin 4·4 mg.%. Coombs ++++. Slight jaundice with mottling of skin. Hepato-splenomegaly +. E.T. 850/800 c.c. Condition poor at end of exchange transfusion, but improved. On 3rd day respiratory difficulty, and oliguria and haematuria and oedema of legs developed; still jaundiced. On 4th day skin petechiae, haemoptysis and death. P.M.—Pulmonary and bladder haemorrhage; kernicterus.

Case C

Second child. B.W. 8 lb. Cord Hb. 15·7 g.%. Serum bilirubin 3·6 mg.%. Coombs ++++. Splenomegaly ++. E.T. 700/700 c.c. Increasing jaundice from 2nd day. Kernicterus and death on 4th day. P.M.—Kernicterus and pulmonary haemorrhage.

Case D

First 2 children normal. Third child given exchange transfusion.

Fourth child. B.W. 7 lb. Cord Hb. 7·2 g.%. Cord bilirubin 6·2 mg.%. Coombs ++++. Jaundiced with ecchymoses on face and back. General condition poor. E.T. 700/700 c.c. Deteriorated during transfusion. Died 18 hours after birth. P.M.—Hepato-splenomegaly; scattered pulmonary haemorrhages; no kernicterus.

Case E

First two children normal. Third and fourth children stillborn.

Fifth child. B.W. 7 lb. 10½ oz. Capillary Hb. 10·9 g.%. Cord bilirubin 10·8 mg.%. Coombs ++++. Deeply jaundiced. Splenomegaly ++. General condition poor. E.T. 730/730 c.c. Deterioration during procedure, with temporary improvement later. Died 20 hours after birth. P.M.—Cerebral haemorrhage right ventricle; left hydronephrosis with grossly dilated ureter.

Case F

First, third and fourth children alive. Three stillbirths and 2 miscarriages.

Seventh child. Elective Caesarean section at 39 weeks. B.W. 4 lb. 14 oz. Cord Hb. 8·0 g.%. Cord bilirubin 9·1 mg.%. Coombs +++. Jaundiced. Hepato-splenomegaly ++. E.T. 425/405. Condition improved after transfusion. Grunting respiration with blood-stained mucus oozing from mouth 7 hours afterwards. Fine crepitations heard at left lung base. Died 12 hours after transfusion. P.M. refused.

Case G

Maternal antibody titre—2,048 (incomplete).

Second child. B.W. 6 lb. 11¾ oz. Cord Hb. 16·9 g.%. Cord bilirubin 4·4 mg.%. Coombs ++++. No jaundice or hepato-splenomegaly. Exchange transfusion not done. Slight jaundice first noticed 36 hours after birth, which then increased. Signs of kernicterus on 4th day. Died on 6th day. P.M.—kernicterus and pulmonary haemorrhage.

Case H.

Maternal blood-transfusion 6 years previously.

Second child. Antibodies not detected antenatally. B.W. 7 lb. 12½ oz. Cord Hb. 4·4 g.%. Cord bilirubin 3·5 mg.%. Coombs ++. Antibodies type anti-c detected—titre 64 (incomplete). Died 20 minutes after birth. P.M.—intracranial haemorrhage; liver and spleen +; scattered haemorrhages, especially retroperitoneal; jaundice; no kernicterus.

SUMMARY

The procedure adopted at the Natal Rhesus Unit, centred at Addington Hospital, Durban, is described from its inception in 1951 to 1955.

The number of cases of haemolytic disease of the newborn encountered during this 4-year period is shown, and the indications for exchange transfusion are discussed.

Results obtained in infants subjected to immediate treatment and in those left untreated are shown, the over-all mortality for the series being 10%.

The incidence of kernicterus is described.

Reference is made to the progress of affected infants

after leaving the hospital, the majority having been followed up for at least 6 months.

Short summaries of the fatal cases are included.

We wish to record our thanks to Dr. J. V. Tanchel, Medical Superintendent of Addington Hospital, for permission to publish this report; to Mr. Harold Renton, Senior Visiting Obstetrician to the hospital, for maintaining faith in our judgement of these cases; and to Dr. J. C. Thomas who, as Director of the Durban Blood Transfusion Service, organized the Rhesus Laboratory Service.

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Paper 7

Central African Journal of Medicine 1958

55.

THE SEROLOGICAL DIAGNOSIS OF HAEMOLYTIC DISEASE OF THE NEWBORN.

by Miss P. Moores. Lecture given 30th April, 1958.

It had been known for a very long time that every now and then a newly born infant suffered an early death with symptoms of deep jaundice and severe anaemia, but the cause remained a complete mystery until only about eighteen years ago. Neither of the two terms used to describe the disease is particularly good. "Haemolytic Disease" correctly presupposes the destruction of red blood cells in vivo, but "of the Newborn" is wrong as the destruction in fact begins in utero. "Erythroblastosis Foetalis" on the other hand, while describing the blood picture in severe cases, does not apply in the milder forms of the disease where erythroblasts are not by any means always present. In addition, erythroblasts can also be present in other disorders, namely infants born to diabetic mothers.

Affected infants show some degree of anaemia at birth, become jaundiced either immediately or within the first twenty-four hours, and the cord is almost always somewhat icteric. The liver and spleen are often enlarged and the skin may show petechial haemorrhages. The jaundice progresses rapidly and the rising serum bilirubin may very soon involve the brain and produce the condition known as Kernicterus. Rhythmic crying or grunting sets in and the spine may become rigid. Death follows swiftly unless some treatment intervenes. These infants are subject to a massive destruction of their own blood cells which is usually too great for their own organs to cope with. Many are exposed too long in utero to the process and are miscarried or stillborn; or may be hydropic at birth which is a condition of extreme anaemia. In almost all cases the placenta is characteristically large and oedematous.

Small wonder that doctors and scientists wished to discover the cause of the disorder.

Karl Landsteiner, in 1900, started the ball rolling by his brilliant discovery of the ABO blood groups. He was able to show, by using his fellow workers as guineapigs, that not all bloods could be mixed together without some reaction taking place. This reaction took the form of strong clumping together of the red cells and even of their complete or partial haemolysis.

Four types or groups were recognised and given the letters A. B. O.

and AB. Each was clear cut and shown by family tree studies to be inherited in a straightforward manner. For example - a Group O woman married to a Group A man would not have any Group B children. Similarly two Group B parents would not have any Group A children unless the legitimacy of the offspring was in doubt. In fact if there were indeed some of Group A then legitimacy was immediately doubted.

The blood group of an individual is inherited in the nature of two genes - one from each parent. A gene is a minute particle of nuclear material and there are many hundreds of them inside each cell nucleus. Each expresses, or helps to express a body character - such as eye colour, shape of the head, texture of the nails and so on, and of course the blood group. Some genes are recessive and masked so that the character does not appear, and some are dominant.

When a cell divides into two daughter cells in the normal process of growing, the genes line up to form the chromosomes which are present in like pairs - one from each parent. These come together and chromosomes and genes divide lengthways forming bundles of four. One member of each split pair then goes to each daughter nucleus which thus receives an exactly similar number as had the original nucleus.

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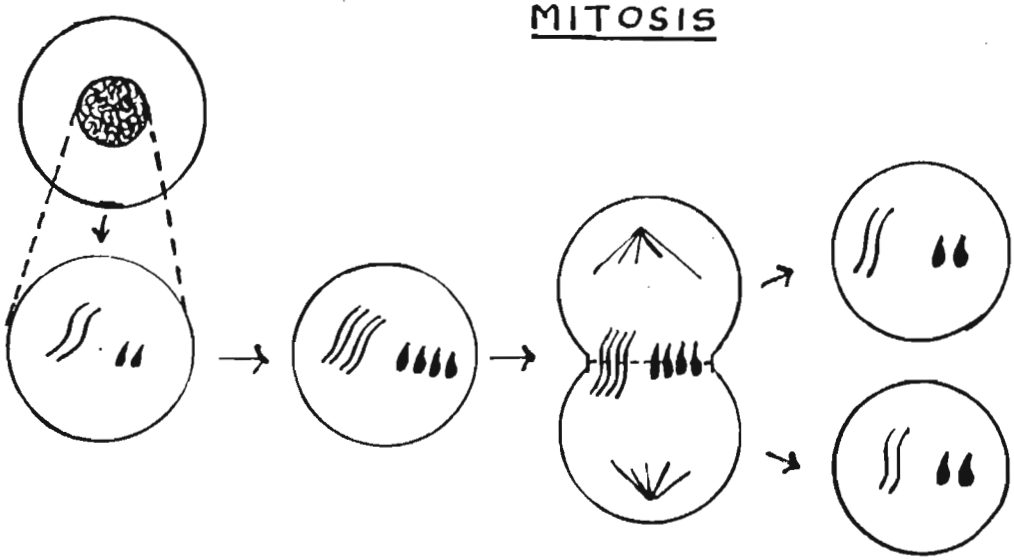
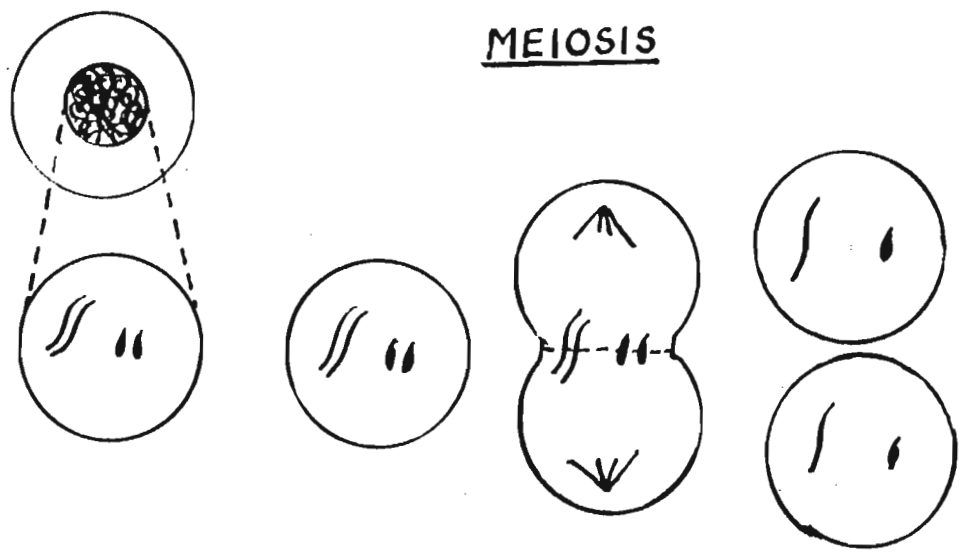


When the sex cells are formed, however, the process is a little different. The genes and chromosomes line up in pairs again but no splitting occurs. Instead the pairs separate and one only goes to each daughter nucleus, which thus has only half the normal number of chromosomes. When such a cell is fertilized it meets another sex cell also with only half the normal number and they fuse re-establishing the normal number again.

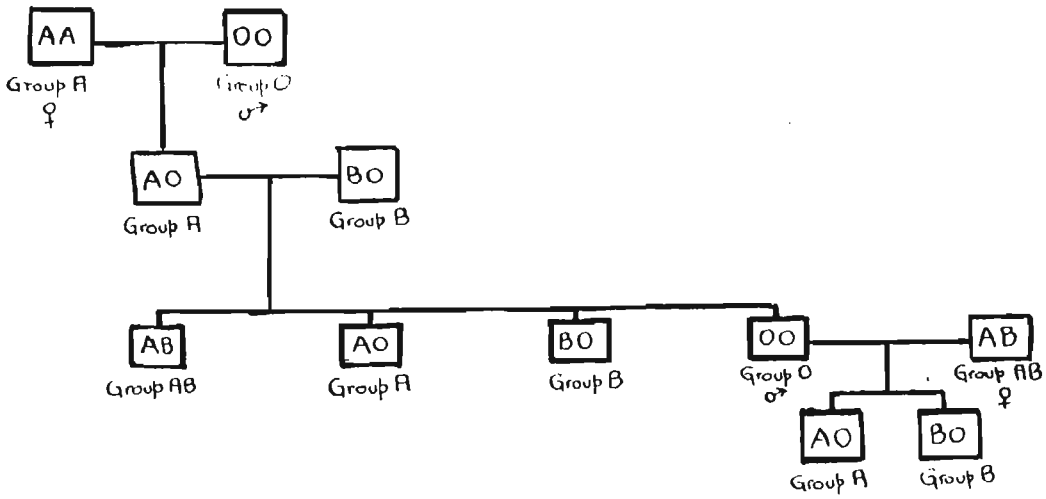


The blood group therefore conforms to the pattern - being a gene. Every red cell of the body has the same group, but in the sex cells the inherited pairs from the parents are re-separated - meet other separated chromosomes and combine again to express perhaps a different blood group.

Group O is always a recessive gene and will only appear when two Group O genes are present - one on each inherited chromosome. If one gene is either A or B, the presence of the O gene is masked and the Group is A or B. When the two dominants A and B are present together the Group is AB.

Here is a typical family tree :-

58.



The serum of a Group A person almost always contains the antibody anti-B. This is effective in destroying the foreign protein of Group B should that gain entry into the circulation at any time. Group B persons have anti-A, Group O have anti-A and anti-B, and Group AB have neither or they would obviously destroy their own cells.

The antibody is usually present from two to three months after birth, and being a saline agglutinin, works best at temperatures lower than 37°C; the optimum being about 4°C. Being of the non-γ globulin type it does not pass through the placenta easily. In certain cases, however, it can take on <u>immune</u> characteristics and the optimum temperature is then altered to 37°C. The power of haemolysing red cells is acquired and the antibody is potentially dangerous as it is now able to pass across the placenta and attack the red cells of a foetus in utero. Anti-A and anti-B become immune when exposed to the corresponding antigen. There are the obvious cases where blood of a different group may have been administered accidentally as in the prophylaxis for measles, or the inoculation against typhoid or diphtheria - very common nowadays - where animal serum rich in an antigen similar to that of the blood groups is injected. Also, a Group AO mother carrying a BO child whose B gene has been inherited from its father, may receive accidentally a small quantity of blood from her child in utero and thus cause her anti-B to become immune. In the majority of cases mothers who develop immune anti-A or anti-B are Group O. The unborn child is not necessarily affected however. Though the immune antibody can pass through the placenta more easily, this does not always occur and only in

1 in 2 to 3,000 pregnancies does the passage cause enough damage to warrant any treatment. In such cases jaundice may appear within the first twenty-four hours as often as it does with Rhesus immunisation, but it is usually mild and starts about the second to third day after birth.

In the Laboratory, some time prior to the expected date of delivery, the serum of all pregnant mothers is examined by a routine screen test for the presence of immune anti-A and/or anti-B. If either is found to be present, the husband's blood is requested in order to determine whether his group is the corresponding antigen or not. For example - if he is Group B or O and his wife has an immune anti-A there need be no concern as she will not have a Group A child by him, and she will have acquired her immune anti-A by another of the ways already mentioned.

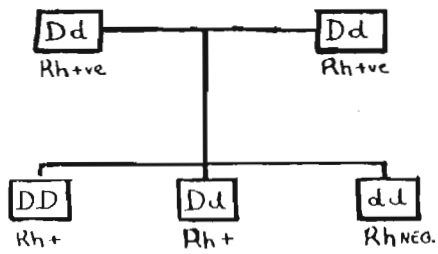
If though, the husband is Group A, the risk to the unborn child is immediately apparent. The Laboratory then determines the optimum temperature of the anti-A against the husband's cells, and a message is sent to the doctor to send cord blood for investigation as soon as possible after the delivery has taken place. When the blood is received the baby's blood group is determined as, should it be any other group except A, there need be no further concern. Assuming then, that the group is the one against which the mother has an immune antibody, the haemoglobin is estimated. This is often normal, but if low, will indicate some anaemia due to cell destruction. A direct Coombs test is performed using serial dilutions of the Anti-Human Globulin. In almost all cases of ABO incompatibility the cells are only weakly sensitized and are more easily agglutinable by diluted Coombs serum. This test may however be quite negative. A serum bilirubin test will indicate clearly whether cell breakdown has been going on, and any figure above 2.8mgms % will necessitate an exchange transfusion as soon as possible. Almost the only other signs of abnormality are a raised osmotic fragility test and the complete lysis of the cord cells at 37°C by the mother's serum, though examination of a stained blood film may show numerous red cell precursors, spherocytes, polychromasia and other signs of rapid cell breakdown and regeneration.

It was not until 1939 - 1940 that the Rhesus Factor entered the field of Blood grouping, and proved to be the major cause of Haemolytic Disease of the Newborn. Landsteiner and Wiener discovered it by the experimental injection

of Rhesus monkey blood into guineapigs which were found to produce an antibody in their serum capable of agglutinating the red cells of 85% of the white population of New York. This later became known as Anti-"Rh." or "Anti-D." Levine and Stetson very quickly showed that the guineapig antibody was the same as that found in the serum of mothers who had had an infant suffering from Haemolytic Disease of the Newborn. It is Rhesus negative women whose husbands happen to be Rhesus positive who are most concerned, though it has been found that on the average, only one in every two hundred pregnancies may expect trouble from the presence of Anti-D. Most Rhesus negative women, despite many pregnancies never experience any trouble at all though the majority must have Rhesus positive husbands. The few who do, provide a useful source of human Anti-D which is used to determine an individual's Rhesus Group nowadays and not guineapig serum. However, the American Blood Banks also have a supply of male volunteers who have been immunised deliberately.

The D antigen is inherited in a straight forward manner as a dominant Mendelian character. As in the ABO groups, the D is a gene carried by a chromosome within the cell nucleus. Each nucleus has two "like" chromosomes (one from each parent) both of which have a D gene, and either or both of which may be Rh. positive or Rh. negative. Thus - if identical - DD or dd, and if dissimilar Dd.

It is quite possible for two Rhesus positive persons to have a Rhesus negative child, in the following manner. The "D" gene is always



dominant over the d gene and thus all persons with either one or two D's are Rhesus positive. If one parent is DD or homozygous, all his children will be Rhesus positive, as he can only pass on to them the dominant D gene. If neither parent has a D gene, none of the children will have it either.

When the D gene is absent and blood containing the D gene is introduced -

in however small a quantity - this acts as a "foreign protein" and the body reacts by developing straight away a protecting antibody which will destroy the protein. That this does not by any means always occur is well known, but it may, and must therefore be guarded against. The antibody Anti-D is almost never naturally occurring, in direct contrast to the Anti-A and Anti-B of the ABO blood groups. There has to be some stimulation for it to appear. Most Rhesus negative people never make Anti-D either because they are never exposed to the antigen, or are reduced in their ability to make antibodies in some way.

When a Rhesus negative mother starts having a family, unless she has been unlucky enough to have had injections of Rhesus positive blood previously her first Rhesus positive child will be normal, but it is from this child that she may receive the small quantity of blood which could cause her to start producing Anti-D. That the blood of a foetus can enter the maternal circulation, probably via the placenta, was shown by Dunsford who found a mother - Group O Rh. negative - with Group A Rh. positive red cells present as well. At birth her baby proved to be in fact Group A Rh. positive.

Once the Anti-D has been found all subsequent Rhesus positive children will be affected. Being a γ globulin it passes through the placenta freely and attacks and destroys the red cells within the unborn child, causing severe anaemia.

There are two types of Anti-D - "complete" and "incomplete". The complete form is a saline agglutinin, and the incomplete acts in albumen; with cells which have been treated with trypsin; or by indirect Coombs test. Both have their optimum temperature 37°C. The Coombs test makes use of a specially prepared rabbit serum anti-human globulin which will agglutinate cells coated with human globulin, i.e. with antibody.

In the Laboratory, the ABO and Rhesus group of every pregnant mother is determined. If she is found to be Rhesus negative her serum is tested by a number of methods against a panel whose Rhesus groups are known. In the majority of cases these tests are negative and a further specimen of her blood is requested for examination about six weeks before term. Antibodies may still appear after this time but will be unlikely to affect the child severely enough for treatment to be necessary. If it is possible for the husband's blood to accompany that of his wife's to the Laboratory, the best test of all is her serum

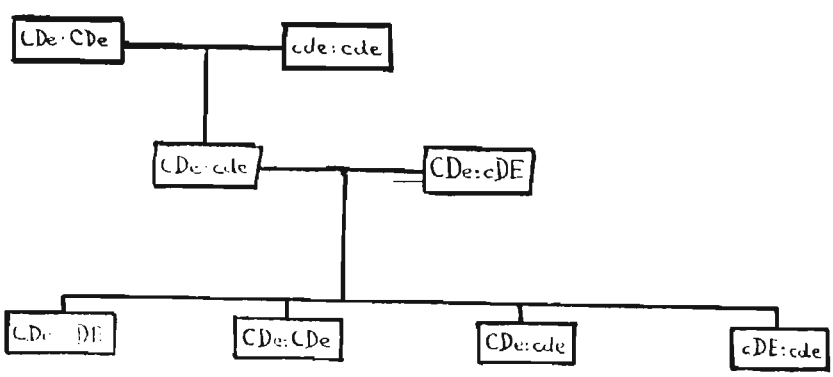
against his cells, for the child will inherit his blood group characters and these are the antigens to which his wife may develop antibodies.

Though Anti-D accounts for 95% of cases of Haemolytic Disease of the Newborn, the other 5% are made up of rare antibodies belonging to the nine known blood group systems. The incidence is very low, but the same principle applies - the wife lacks the antigen and develops an antibody against her child's cells whose antigen has been inherited from its father. Some antibodies are so rare that only one family has ever been known to possess them, and the collection and identification of these is the major concern of many leading Research workers. For example - the Rhesus System itself is composed of eight related genes occurring in four pairs in the cell nucleus. Each set of four is inherited as a unit from a parent, and are not separated. The units are as follows:-

Common.	Rare.	Very Rare indeed.
CDe F	Cde f	CdE F
cDE F	cdE f	
cde f	CDE F	
cDe f		

The Ff genes are the most recently discovered and so far there is no known anti-F or anti-f. However, antibodies are known against all the other genes and may also be present as a mixture, such as Anti-CD or Anti-DE

An example of a family tree showing the inherited units follows:-



Subgroups of the Rhesus genes are also known, and of these the most common is D^u. Though often undetectable using an anti-D of the "complete" type in grouping, its presence can be established by using an incomplete anti-D followed by a Coombs test. It is important especially in the grouping of Blood Donors to know whether an apparent Rhesus negative blood is D^u positive or not, as such a blood might stimulate the production of anti-D in a Rhesus negative person. Also a D^u positive is capable of developing anti-D. No blood may be said to be truly Rhesus negative unless it has been tested with anti-C, anti-E, and for D^u, with negative results.

As soon as Rhesus antibodies are detected in a pregnant mother, they are identified and titrated to determine the degree of activity reached. Titrations, however, may often be misleading, and an avidity test is a better guide. * A plan of action is then decided upon.

Account of the previous obstetric history is taken; any rise or fall of the titre or avidity is noted, particularly if it occurred towards the latter end of pregnancy; and the possibility of the husband being homo- or heterozygous is studied. Mild cases and those likely to have a Rhesus negative child are allowed to deliver normally at full term. If however, the indications are that the child will be affected labour may be induced three to four weeks before term, or there may be an elective Caesarean section. Both are aimed at removing the child from the active danger at the earliest possible moment. Once the child is born it is necessary to find out whether an exchange transfusion is needed or not, and cord blood is examined as quickly as possible in the laboratory. The haemoglobin, blood group, direct Coombs test and serum bilirubin are investigated. If the Coombs test is positive, the haemoglobin below 14.8 gms % and/or the bilirubin above 2.8 mgms %, an immediate exchange transfusion must be done. Two fresh pints of blood of the child's own ABO group and of a Rhesus group which will not be affected by the antibody are cross-matched without delay and then partially concentrated by the removal of some of the plasma to raise the relative haemoglobin from normal adult to nearer that of a normal newly born infant. This blood should tide the child over for two to three months and prevent it making its own Rhesus positive cells until nearly all the retained antibody has been removed or destroyed.

For an exchange transfusion the child is placed on a raised bed and kept warm. The cord is cut one to two centimeters from the skin and a

* Personal communication of A.L.C.HUNT.

polythene catheter which has been dipped in heparin is carefully inserted into the umbilical vein. Once the catheter is in place, a 10 cc. syringe is connected and blood is alternately withdrawn from the child, discarded, and donor blood injected in its place. This process continues until the child has received 70 to 80 ccs. per lb. of body weight. The purpose of the exchange is to remove as quickly as possible the circulating serum bilirubin which may have reached a dangerously high level with a risk of Kernicterus. It also removes retained antibody and sensitized red cells, substituting cells which will not be affected.

It is of the greatest importance to test all pregnant mothers for the presence of blood group antibodies. One can then be ready to perform the necessary tests on the baby, and prepare an exchange transfusion with the minimum of delay when necessary.

If the mother's group is unknown and no ante-natal tests have been done, there must inevitably be delays which might very easily prove fatal to the child. For the maximum effect an exchange transfusion should be begun at the earliest possible moment after delivery.

It is also of extreme importance that good cord blood samples be taken and the tests performed with the maximum of speed. A clotted cord haemoglobin sample means that the true figure is forever lost, as capillary or venous blood taken later give a confusingly higher result.

The first child is usually unaffected, but once antibodies are developed all subsequent children will suffer though not necessarily all to the same extent. If the husband is heterozygous, i.e. Dd, unaffected Rhesus negative children may appear between affected ones. Also a child inheriting the unit CDe is on the average less severely affected than one inheriting cDE. On the average, however, each successive pregnancy is progressively more affected than the last.

It is often asked whether Rhesus antibodies might appear in the mother's milk and be a source of continued cell destruction in the child, but though Grifols of Spain has recovered a very low titre antibody from a Rhesus negative baby fed on milk containing antibodies of a very high titre, he was not able to detect any sensitization by direct Coombs test of a Rhesus positive baby's cells when treated similarly.

As an example of the lengths one has to go sometimes in blood grouping,

there occurred elsewhere recently a pregnant mother with a very rare blood group indeed, known as Tj a negative. She was also Rhesus negative and had a very potent anti-Tj a. The only compatible blood was to be found in a woman in Australia who, by sheer good fortune was on holiday in the country at the time. Some correspondence located her and a pint of her blood was taken and kept frozen in glycerol until the delivery. It was the only known compatible pint in the world. *

With this in mind we are very fortunate that for practical purposes we need only concern ourselves with the possibilities of ABO and Rhesus incompatibility and leave the museum pieces to Research workers in other places.

* Personal communication of M. Cuthbush.

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Paper 8

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The Incidence and Severity of Jaundice in the Newborn in Salisbury, Southern Rhodesia

BY
A. L. C. HUNT, PHYLLIS MOORES
AND
D. W. PLAMPIN
Dr. G. V. Blaine's Laboratory, Salisbury, S. Rhodesia.

Introduction

It became apparent during 1959 and 1960 that the numbers of newborn babies requiring transfusion to prevent kernicterus in Salisbury, S. Rhodesia, were far above those reported elsewhere.

Mollison (1956) states: " . . . Haemolytic disease of the newborn due to ABO incompatibility has a minimum incidence of one in 150 births; the word minimum is used because the estimate is based on the proportion of infants who develop jaundice. It may be taken as certain that other infants have a mild haemolytic process due to ABO incompatibility, but never display jaundice. However, only one in 2,000 or less of all newborn infants have a sufficiently severe degree of haemolytic disease of the newborn due to ABO incompatibility to require treatment."

Weiner, Wexler and Hurst (1949) say that when the haemolytic disease is due to ABO incompatibility the infant as a rule is not seriously affected.

Valentine (1958) states " . . . that haemolytic disease can occur in clinically recognisable degree as often as once in every 71 births," and describes a series of 14 cases where three were sufficiently affected to require exchange transfusion.

Cunningham (1959), however, from a series of investigations on babies suffering from "physiological jaundice," did not support this view. He also showed that from 1,000 babies investigated, none at full time with normal weight required transfusion and only one (weight 2¼ lb.) required transfusion from physiological jaundice.

In view of the former findings it was decided to correlate results obtained in the laboratory for the years 1959 and 1960.

(1) To establish whether there is an increased incidence of haemolytic disease of the newborn due to immune anti-A or B.
(2) To show whether there is an increased severity of the disease in this area.
(3) To emphasise the need for adequate antenatal serological investigation of all women, irrespective of Rh group or number of pregnancies.
(4) To provide a scheme of investigation for all jaundiced babies so that the antibody involved, if any, may be identified quickly and compatible blood provided in the shortest possible time.

Materials and Methods

Throughout the period under discussion the sources of grouping sera and the methods used in all tests remained constant.

Groups A, B and O sera were supplied by the South African Institute for Medical Research, Johannesburg. The various Rhesus grouping sera and the Coombs reagent were obtained from the Certified Blood Donor Service Inc., Jamaica 35, New York. The 20 per cent. bovine albumin solution used in titrations, compatibility tests for exchange transfusions and in ante-natal tests was obtained, sterile, in 20 ml. quantities, from Armour Laboratories (Armour & Co. Ltd.), Hampden Park, Eastbourne, England. Merck, Sharpe & Dohme (Division of Merck & Co. Inc.), Philadelphia, P.A., supplied the blood group specific substance A and B for neutralisation tests used in ante-natal and cord blood investigations. The Salisbury and District Blood Transfusion Service donated regular samples of blood from European donors who had all been genotyped as far as possible. This provided a satisfactory panel of blood group antigens for ante-natal antibody screen tests and in investigations involving the identification of an unknown antibody.

The estimation of cord blood haemoglobin was made on an M.R.C. wedge photometer which was kept constantly checked.

The bilirubin estimation employed was that of Malloy and Evelyn (1937), Watson (1946) and Gray (1947), readings being made on a Hilger biochemical absorbtionometer.

(a) *Ante-Natal Tests*

All investigations over the whole of the two-year period were made essentially on women of European descent. Each patient was grouped for ABO and Rhesus (D) by the methods on

pages 140 and 161 of Dunsford and Bowley's *Techniques in Blood Grouping*, 1955. Those that were found to be Rhesus (D) negative were further tested for the antigen Du (Dunsford and Bowley's Technique No. 55).

All specimens of serum whether from Rhesus negative or Rhesus positive patients, were then screened for the presence of haemolysins against A and B cells (Dunsford and Bowley's Technique No. 45 iii). Other blood group antibodies were tested for in saline at 37° C., in 20 per cent. bovine albumin solution at 37° C., and by indirect Coombs test (Dunsford and Bowley's Technique No. 65), against a panel of cells of known groups. Tests in saline at room temperature were not made except in special circumstances where the antibody detected appeared to have an optimum degree of agglutination at a temperature lower than 37° C.

In cases where the haemolysin test was positive the husband's blood was obtained and the blood group determined. When this proved to be of a group unrelated to the wife's haemolysin her serum was tested directly against her husband's cells in saline at 37° C., 20 per cent. albumin solution at 37° C. and by indirect Coombs test to exclude all abnormal antibodies. When, however, the group did correspond to the haemolysin, the wife's serum was subjected to a thermal optimum test against her husband's cells and a neutralisation test, using blood group specific substance A and B (Dunsford and Bowley's Technique No. 45). If either of these were positive her doctor was advised accordingly and cord blood was requested as soon as possible after delivery.

(b) *Cord Blood Tests*

Haemoglobin. It was found that in infants affected with haemolytic disease of the newborn due to immune anti-A and anti-B, the cord haemoglobin levels were almost always above the average range (normal average range is 15-18 gm. per cent.).

Bilirubin.—The normal range of bilirubin in cord serum is accepted as 0-2.8 mg. per cent. Where the antibody was immune anti-A or anti-B, bilirubin tests were performed daily, and exchange transfusion was given only when the level neared or exceeded 20 mg. per cent.

Group and Rhesus. Dunsford and Bowley's Techniques Nos. 38 and 50.

Direct Coombs Test.—Cells from the cord were washed three to four times in normal physiological saline and a Coombs test done. The Coombs serum was diluted serially from 1/1 to 1/256 in order that an optimum dilution might be routinely present. This was often found helpful in cases of immune anti-A and anti-B sensitisation where the agglutination tended to be stronger in the 1/2 to 1/8 range of dilutions.

Mother's Serum Against Baby's Cells.—When the baby's cord cells gave a negative direct Coombs test the mother's serum was tested directly against them in saline at room temperature and at 37° C., in 20 per cent. bovine albumin solution at 37° C. and by indirect Coombs test as a final check for antibodies. An avid immune anti-A or anti-B in the mother's serum rapidly haemolysed the baby's cells (if appropriate group), in saline at 37° C. in under 30 minutes, and complete or partial haemolysis was taken as an indication of the degree of avidity of the haemolysin.

(c) *Exchange Transfusion*

Fresh compatible donor blood was concentrated just before use by centrifuging, and approximately 100 c.c. plasma removed. It is realised that the use of washed cells might have been preferable, particularly in the case of infants affected by immune anti-A or anti-B, but at present it is not possible to effect this in Salisbury. In ABO affected cases Group O blood was used, with the Rhesus group corresponding to that of the infant and especially selected for low titre non-immune anti-A or anti-B. Where the jaundice appeared to be due to physiological causes, blood of the infant's own group was chosen. Bilirubins were estimated on specimens taken from the cord both at the commencement and at the termination of the exchange transfusion, but all later tests were made on serum obtained from blood taken from heel puncture.

Discussion

The statistics quoted were from full-term infants, all of normal average weight (6-9 lb.), so that prematurity is not a factor that influenced the increased severity of these cases.

It is apparent that the numbers of babies requiring transfusion for jaundice other than that due to Rh antibodies (Table I) are far in excess of the numbers reported by Mollison, Weiner or Valentine, although the ratio of babies affected with haemolytic disease of the newborn due to immune anti-A or anti-B to the total births compares favourably with Valentine and Mollison's figures (Table I).

The incidence and severity of "physiological jaundice" is greater than that reported by

Cunningham. The disease is more severe in Salisbury (S.R.) than is encountered elsewhere.

The infants transfused in the ABO and physiological groups had bilirubins of between 18 and 28 mg. per cent. before exchange transfusion was done. All had been watched clinically from birth and regular bilirubins estimated until it was apparent that the increasing bilirubinaemia was approaching the danger zone for the development of kernicterus.

It is interesting to note that the number of infants with severe hyperbilirubinaemia requiring blood transfusions, caused by ABO haemolytic disease and "physiological jaundice" (Table II), is greater than among those infants requiring blood transfusion from haemolytic disease of the newborn due to Rh antibodies (Table II).

Using Coombs reagent in optimum dilution, it has been found that 44 out of 44 cord bloods were Coombs positive in cases of haemolytic disease of the newborn due to immune anti-A or anti-B. With the system of investigation as illustrated in the representative case shown, there

Table I

	Number of Live Births	RH Babies	ABO Babies	Physiological Jaundice
	5,425	39	44	27
Ratio to live births		1: 139	1: 123	1: 201
Normal expected ratio		1: 200	1: 75	Nil full time (Cunningham)
Requiring transfusion		37	26	19
Ratio to live births requiring transfusion		1: 147	1: 208	1: 285
Normal expected ratio to live births requiring transfusion		1: 300 (Mollison)	1: 2,000 (Mollison)	Nil full time (Cunningham)

Table I shows the number of live births in Salisbury, Southern Rhodesia, during the period January, 1959, to December, 1960.

The number and proportion of infants requiring transfusion is compared with those expected from previously accepted statistics (Mollison; Cunningham).

Table II

Type of Jaundice	Total Numbers	Number Needing One Exchange Transfusion	Per Cent.	Number Needing Two or More Exchange Transfusions	Per Cent.	Total Per Cent. Needing Transfusion
RH	39	25	64.1	12	30.7	94.8
ABO	44	20	45.4	6	13.6	59.0
Phys.	27	14	51.8	5	18.5	70.3

Table II shows the percentage of each groupprequiring exchange transfusion.

Table III

Total Number of Jaundiced Infants	Total Number Transfused	Transfused for Rh Antibodies	Transfused for ABO and Physiological
110	82	37	45

Table II

Maximum Total Bilirubin	ABO	Physiological
16-18 mg. per cent.	2	—
18-20 mg. per cent.	6	1
20-22 mg. per cent.	6	6
22-24 mg. per cent.	5	5
24-26 mg. per cent.	4	3
26-28 mg. per cent.	3	4
	26	19

Case History

Mrs. S—

Antenatal: Group "O" Rh positive (D).

Rh antibodies: Nil. (Panel of known cells: Saline 37° C., 20 per cent. albumin 37° C., Trypsin, indirect Coombs test techniques.)

Haemolysin test: Positive against A cells.
Husband's group: A Rh positive (D).
Thermal optimum test against husband's cells—

	4°	22°	37°
Titre	64	128	512

Immune anti-A.

Cord blood: Haemoglobin = 145 per cent. = 26.46 gm. per cent.

Direct Coombs test (Coombs serum in serial dilution): Positive 1/2 to 1/16.

Group "A" Rh positive (D).

Bilirubin: Total, 3.1 mg. per cent.: indirect, 2.5 m. per cent.: direct, 0.6 mg. per cent.

	Saline R.T.	20 per cent. Albumin 37° C.	Haemolysin 37° C.
Mother's serum x baby's cells	+	+	++

Mother's serum neutralised with A and B specific substance—

	Saline 4°	Albumin 37° C.	Indirect Coombs	Haemolysin
x baby's cells	—	—	+*	—
x O panel	—	—	—	—
x A cells	—	—	+	—
x B cells	—	—	—	—
x husband's cells	—	—	+	—

Baby's bilirubin (mg. per cent.)—

	Second Day	Third Day	Fourth Day
Total	8.2	15.8	20.1
Direct	0.8	1.2	1.0
Indirect	7.4	14.6	19.1
	Post Transfusion	Fifth Day	Sixth Day
Exchange transfusion given	9.4	14.6	11.0
	0.6	0.9	0.6
	8.8	13.7	10.4

* If the baby's cells are positive by direct Coombs test, this reading will be positive in any case and may be left out of the series.

should be no difficulty in conclusively demonstrating the presence of active immune anti-A or anti-B.

From the statistics given in Figs. 1, 2, 3 and 4 there is some doubt as to whether the ABO-affected infants requiring transfusion may not in fact have superimposed "physiological jaundice" as well.

The reasons for the higher incidence of severity of ABO haemolytic disease of the newborn and of "physiological jaundice" in Salisbury may be accounted for in at least two ways.

(1) *"Physiological Jaundice"*

The average haemoglobin estimation in this group ranges between 18.07 gm. per cent. and 22 gm. per cent.

The possibility of dehydration was considered during the course of investigation, as on these babies it was noted on many occasions that the bilirubin concentration fluctuated in unison with the clinically assessed degree of hydration or dehydration.

The regime adopted at the maternity hospital was found to be:

From Birth—

3-5 lb.: No fluids for 48 hours.
5 lb. +: No fluids for 24 hours.
Then 2 oz. (approximately) of total fluids per lb. body weight is given in 24 hours.

This is less than the amount given in temperate climates (15-19 oz. in 24 hours). Also the nursery is mainly glass enclosed and average temperature throughout the year is 78°-80° F. It is suggested that the fluid intake of these babies is insufficient, especially as the humidity in Salisbury (Meteorological Office) is sometimes as low as 10 per cent. and the average summer temperature range 70°-90° + and in winter mean temperature of 56.5°.

(2) *Immune Anti-A and Anti-B.*

The majority of the adult European population in S. Rhodesia receive inoculations such as T.A.B., yellow fever and smallpox vaccination on repeated occasions. This may stimulate the production of all types of antibodies, including the ABO antibodies. From Table I it may be noted that the ratio of Rh-affected infants (1: 139 of all pregnancies) is high and the percentage (94.8 per cent.) requiring exchange transfusion is greater than encountered elsewhere. Whether this may be explained by the increased heterologous antibody production due to inoculations is a matter for conjecture.

As stated earlier, the possibility of superimposed "physiological jaundice" due to dehydration must always be considered, although for the purpose of exchange transfusion it is advisable to treat the individual case as being ABO-affected.

Conclusions

(1) It has been shown that the jaundice among full-term infants of normal birth weights in Salisbury (S.R.) is far greater than that reported by Mollison, Weiner and Cunningham in other countries.

(2) Although the incidence of ABO-affected babies is less than that reported by Valentine, the proportion of infants with sufficient jaundice requiring exchange transfusion is far higher.

(3) Hypotheses have been put forward in an attempt to explain this high incidence and severity of jaundice.

(4) It could not be proved that the severity of jaundice in the ABO-affected infants was entirely due to immune anti-A or anti-B, or whether "physiological jaundice" of dehydration origin was a contributory factor.

(5) (a) To conclude, we should like to emphasise and confirm the findings of Valentine: that infants should be closely watched for haemolytic disease of the newborn due to immune anti-A or anti-B and not simply dismissed as cases of "physiological jaundice."

(b) We would go further and say that in Central Africa all European women, whether Rhesus positive or Rhesus negative, should be checked ante-natally for antibodies and haemolysins, and if the presence of immune anti-A or anti-B is found and the husband is of significant group, the cord blood of the baby should be investigated at birth.

(c) That dehydration should be seriously considered as a cause of the increased bilirubinaemia in all groups of jaundiced babies reported in this paper.

Acknowledgments

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Paper 9

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The Third Example of Anti-hr^S

B. G. GROBBELAAR, M.D., B.CH., P. P. MOORES, B.SC.
The Natal Blood Transfusion Service, Durban, Republic of South Africa

A third example of anti-hr^S, first described by Shapiro in 1960, was detected in the serum of a Bantu woman whose newborn infant suffered from mild hemolytic disease of the newborn.

IN 1960 Shapiro[1] reported a new antibody, anti-hr^S, which he discovered in the serum of a Bantu woman, Mrs. Shabalala. The patient's newborn infant had suffered from hemolytic disease of the newborn, with a positive direct antiglobulin test and rapidly advancing jaundice. Shapiro's work showed that the hr^S factor was part of the Rh-Hr complex, although its exact relationship to the various Rh-Hr factors remains to be elucidated. Shapiro exchanged samples of blood with Dr. R. E. Rosenfield, who had previously found an unidentified high-frequency antibody in the blood of a Puerto Rican woman (Santiago). The bloods of Shabalala and Santiago were found to be virtually identical in their Rh phenotypes, and compatible with each other. The Shabalala and Santiago sera gave identical reactions, and constitute the first two examples of anti-hr^S.

Individuals lacking the hr″ factor, also lack the hr^S factor, so that what is often called anti-hr″, may in fact contain anti-hr^S in a varying proportion, and in some instances, consist solely of anti-hr^S. Similarly most people possessing the hr″ factor also possess the hr^S factor as part of the Rh-Hr complex. In a small percentage of cases, particularly in Negroes, the hr″ factor is present without the hr^S component. In such cases pure anti-hr^S (*i.e.* without anti-hr″) may be formed, and unless tested against cells containing the hr″ factor but lacking the hr^S factor, will be diagnosed as ordinary anti-hr″. If such cells are not available, the presence of anti-hr^S can be suspected from the fact that an antibody of apparent anti-hr″ specificity occurs in an individual whose cells react with standard anti-hr″ reagents. It was circumstances such as these which led to the detection of this third example of anti-hr^S.

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Case Report

Mrs. Sakwe, a Bantu woman aged 24 years, gave birth to an apparently normal infant of 8 lbs. on May 31, 1959. Samples of the cord and maternal blood were submitted to the Natal Blood Transfusion Service for investigation. Antenatal investigations had not been carried out during the course of pregnancy.

Investigations on the cord blood showed it to be group A, Rh_0. The direct antiglobulin test was negative. The mother's serum showed a 1+ macroscopic reaction with the infant's cells at room temperature and at 37 C. No further investigations were carried out at the time. The serum from a donation of blood received from the patient shortly afterwards was stored in a deep-freeze for more detailed analysis at a later date. The infant made an uneventful recovery with the serum bilirubin reaching a maximum of 4.5 mg. per cent on the day after the delivery. The infant did not require treatment, and some doubt remained as to whether it actually had suffered from hemolytic disease of the newborn in view of the negative antiglobulin test.

In November, 1961, the stored serum was re-investigated and the antibody identified as anti-hr″. This seemed strange, as our records showed that her cells had originally been typed as Rh_2rh. Her cells were not available for confirmation of these results, and no further progress was made.

On December 8, 1961, through a stroke of good fortune, we received an antenatal specimen from a Bantu woman who turned out to be the same patient. She was then near term.

The antibody in this specimen was again identified as anti-hr″ and the patient's Rh type was confirmed as Rh_2rh. Her cells reacted weakly with anti-hr″. This reaction was confirmed with three other batches of anti-hr″ diagnostic sera, two of which had been obtained from different commercial suppliers.

There appeared to be no doubt that the patient contained the hr″ factor in her cells, and had produced an anti-hr″ in her serum. Her cells gave a negative reaction with the direct antiglobulin test, and did not react with her own serum in saline, with bromelin, or with the indirect antiglobulin technic, excluding the possibility of an autoantibody.

The provisional diagnosis of anti-hr^S was made. A sample of the serum and cells of the patient were sent to Dr. M. Shapiro of the South African Blood Transfusion Service, who confirmed the specificity of the antibody as anti-hr^S, and the Rh type as $Rh_2\hat{R}h_o$. (Shapiro designated bloods reacting with anti-hr″ but not with anti-hr^S with a caret (^) sign.) In addition he detected an antibody which reacted weakly with papainized Rh_2Rh_2 cells, demonstrating the presence of a similar type of antibody found by him in association with the original anti-hr^S in Mrs. Shabalala, and which he designated as anti-Hr. Cross-reactions were not demonstrable between Mrs. Sakwe's serum and Mrs. Shabalala's cells and vice versa. Shapiro[2] again stated his belief that anti-hr^S is fairly common and that many so-called anti-hr″ sera marketed contain only anti-hr^S, particularly if they were obtained from Negro donors.

The anti-hr^S from Mrs. Sakwe was found to react well in saline giving a 2+ to 3+ macroscopic reaction. This reaction was equally strong at room temperature and 37 C. The reaction was not enhanced, though not inhibited, by the addition of bromelin, and was slightly weaker by the indirect antiglobulin technic.

The infant was born on December 28, 1961. The cells were found to be group O, Rh_o, and reacted with a sample of anti-hr^S kindly supplied to us by Dr. Shapiro. The indirect antiglobulin test was weakly positive (1+ macroscopic), confirmed with several different batches of antiglobulin reagents. The microhematocrit was 52 per cent and the serum bilirubin 1.0 mg. per cent. The infant made an uneventful recovery and did not require treatment.

References

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Serology and Genetics of the Red Blood Cell Factor Rh34

Phyllis Moores, Elizabeth Smart

Natal Institute of Immunology, Natal Blood Transfusion Service, Durban, South Africa

Abstract. Rh34 antibodies were found to have a unique, previously only partly characterised, specificity within the Rh system. No evidence was seen that they were mixtures of hr^B and Hr-like antibodies, or that hr^B antibodies existed independently in the natural state. The term anti-hr^B applied to Rh34 antibodies after they had been partially absorbed with R_2R_2 red cells. Four haplotypes not expressing Rh34 antigen were identified in the present study. The prefix * has been used to indicate them in this text. They were *r^{ss} (*$dCce^s$), *R^o (*Dce), *R^{on} (*$D^{\prime\prime}ce$) and *R^{od} (category III *Dce). Red cells with partially deleted or Rh_{null} phenotypes were therefore not the sole red cells compatible with anti-Rh34. R_2R_2 red cells, which are known to carry weak Rh34 antigen, were incompatible. Twenty-two family and mother-child studies established that the Rh:–34 haplotypes were inherited as normal Mendelian dominant characters. Anti-Rh34 was capable of recognising *RH 34* dosage and of excluding some men who had been wrongly accused in disputed paternity tests.

Introduction

In 1973 the total antibody activity in the serum of Mrs. Bastiaan was assigned the numerical term anti-Rh34 [1]. The previous year, Shapiro et al. [2] reported that Mrs. Bastiaan's serum agglutinated all red cells with normal Rh phenotypes, but failed to agglutinate either partially deleted or Rh_{null} red cells. After these workers had incompletely absorbed a sample of Mrs. Bastiaan's serum with R_2R_2 red cells, they found that it contained e-like antibodies, and they named them anti-hr^B. No further details about the activity of the antibodies in Mrs. Bastiaan's unabsorbed serum were recorded. No accounts of subsequent studies with similar antibodies, nor of Rh:–34 red cells with other than partially deleted, Rh_{null} or the occasional unusual Rh variant phenotype, have been found.

We present a summary of our findings obtained during 26 years of detailed investigations in which 36 sera containing Rh34 antibodies were identified. The findings show that 4 Rh haplotypes determine Rh:–34 red cells. Moreover, these cells otherwise have common Rh phenotypes.

Materials and Methods

The majority of the blood samples were obtained from Natal Black and Coloured (mixed race) blood donors, antenatal patients and transfusion recipients. The remainder were sent by colleagues in South Africa and some neighbouring countries for antibody identification, investigation of unusual Rh phenotypes and our interest.

Standard serological methods were used [3]. The one-stage ficin tile technique, adapted earlier by Dr G.H. Vos (Natal Institute of Immunology, Durban, South Africa) from the method outlined by Simmons et al. [4], proved the technique of choice. It was used routinely to confirm the identity of the antibodies, as their avidity with all the red cell samples of an identification panel was observed easily in parallel. Equal volumes of serum, 2–5% red cell suspension in the preserving fluid of Burgess and Vos [5] and 0.25% ficin solution in phosphate-buffered saline (pH 7.3) were placed in this order on a 280 × 100 mm glass tile. After gentle agitation and hand rotation, the tile was transferred to a moist chamber at room temperature (20–24°C) for 30–60 min. The tile was then tilted to an angle of approximately 40° for 10 s and returned to horizontal over a source of light. The results were all recorded immediately. All negative, weak positive and any questionable results were confirmed by re-tilting the tile and, if necessary, by microscope. Initially, the titrations were also made by this technique. Later, however, one- and two-stage bromelin titrations were substituted to ensure that the titration end-points were clear. The anti-VS

Table 1. Antibodies identified in 54 persons with Rh:−34 or Rh:34w red cells

Antibodies	Antenatal patients		Crossmatch patients		Blood donors		Total
	Rh:−34	Rh:34w	Rh:−34	Rh:34w	Rh:−34	Rh:34w	
Anti-Rh34	9	1	6				16
Anti-C, (weak Rh34)	1	2					3
Anti-Rh34, -D	1	1	5				7
Anti-Rh34, -D, -C, -E			2				2
Anti-Rh34, -D, -Jk^b	1						1
Anti-Rh34, -C	1	1					2
Anti-Rh34, -C, -E			1				1
Anti-Rh34, -E					1		1
Anti-Rh34, -E, -V	1						1
Anti-Rh34, -M, -Le^a				1			1
Anti-Rh34, -HI				1			1
Anti-D	3				2		5
Anti-D, -E			1				1
Anti-E	6		1				7
Cold saline	2		1				3
Insufficient serum	1		1				2
Total	26	5	18	2	3		54

reagent used was Kemper, kindly supplied by Dr. P.D. Issitt. The anti-CD and anti-V which this serum also contained was removed beforehand by absorption. Small samples of Dr. A.S. Wiener's original anti-Rh^A, anti-Rh^B, anti-Rh^C and anti-Rh^D were much valued gifts from Dr. G.H. Vos. The red cells of rare phenotypes and other rare reagents used were from SCARF members, colleagues in South Africa and friends in other countries. Some red cell samples were kindly typed as D category III by Dr. P. Tippett (MRC Blood Group Unit, London, UK). The reagents used in Durban for Rh phenotyping were from the routine laboratory stocks of the Natal Blood Transfusion Service. They had been standardised according to the AABB Technical Manual [3] and the Code of Federal Regulations (1989) of the American Food and Drug Administration.

In this paper, the DCE, numerical and shorthand symbols for the Rh system [6] have all been used, but not necessarily in tandem. For clarity, the Rh:−34 haplotypes and corresponding phenotypes have been prefixed with a * sign.

Results

Survey of the Anti-Rh34 Sera Studied

Table 1 shows the antibodies identified in 47 persons with Rh:−34 and 7 persons with Rh:34w (weak positive) red cells. The sera have been subdivided according to their origin in antenatal patients and transfusion recipients or in blood donors. Thirty-six sera contained anti-Rh34. Seventeen of these also contained other, mostly Rh, antibodies. The anti-Rh34 gave negative results with homozygous *D−−* (1 British and 2 Canadian), homozygous *Dc−* (1 Canadian) and Rh_{null} (1 Japanese amorph and 4 British and USA regulator) red cells. They also gave negative results with Ann Bastiaan's red cells and those of many other subjects whose cells were compatible with Ann Bastiaan's unabsorbed serum. Negative auto-control tests confirmed that the anti-Rh34 had allo- and not auto-antibody specificity. In tests and titrations with antibody identification panel cells, the anti-Rh34 gave a characteristic pattern of reactions (see below). When strong Rh34 antibodies were incompletely absorbed with R_2R_2 red cells, both the eluates and the absorbed sera were found subsequently to contain this specificity. Anti-Rh34 was not identified in any subject who had R_2R_2 red cells.

The red cells of the D+ Rh:−34 subjects who had made anti-D, anti-D and anti-E or anti-D and anti-Rh34, and those of other subjects in whom no antibodies had been detected, were found to have the D type Rh^d (D category III) antigen. Their anti-D was specific for the Rh^D epitope of the D antigen mosaic.

The anti-E and cold saline (anti-H and -HI) antibodies in the Rh:−34 subjects, and the antibodies not identified in them due to insufficient serum, were included in table 1 for interest.

Reaction Patterns

Table 2 shows the characteristic reaction pattern obtained by two-stage bromelin and by ficin tile technique when Rh34 antibodies were titrated against antibody identification panel cells. The C+ cells were regularly aggluti-

Table 2. Characteristic reaction pattern given by Rh34 antibodies in two-stage bromelin and in ficin tile titrations with many red cell samples having the common Rh phenotypes shown above the dotted line

Red cells	Anti-Rh34 dilutions											Score
	1	2	4	8	16	32	64	128	256	512	1,024	
R_1R_1	4	4	4	3	3	3	2	2	1	1	–	92
rr	4	3	3	3	2	t	(3)	(1)	–	–	–	59
R_2R_2	3	2	1	(3)	–	–	–	–	–	–	–	26
R_2R_o	3	3	3	2	2	t	–	–	–	–	–	51
R_2R_o *(R²/*R″)*	2	(3)	–	–	–	–	–	–	–	–	–	t1
$*R_o$ *(*R″/*R″)*	–	–	–	–	–	–	–	–	–	–	–	0
*r″r *(*r″/*r″)*	–	–	–	–	–	–	–	–	–	–	–	0

The genotypes of the R_2*R_o, $*R_o$ and *r″r red cells shown below the dotted line were derternined from family studies (scoring adapted from Marsh [7]).

nated to the highest and the heterozygous R_2*R_o cells to the lowest titres. When red cells with particular phenotypes were agglutinated to higher than expected titres, the sera were almost always found to contain additional, mainly Rh, antibodies. The anti-Rh34 were consistently identified most easily by enzyme techniques. Their titres by bromelin technique with R_1R_1 cells ranged from 16 to 1,024, those with rr and R_o cells from 0 to 128, and those with R_2R_2 cells from 0 to 32. In 31 cases where insufficient serum was available for complete testing, the Rh34 antibodies in 10 of 15 sera reacted by saline and in 12 of 16 sera by indirect antiglobulin technique (IAT). No fully tested sera gave positive results by IAT only. The higher-titred Rh34 antibodies reacted regularly by all three techniques.

The weaker examples of anti-Rh34, which occurred in subjects with Rh:–34 and with heterozygous R_2*R_o or $*r'^sR_2$ red cells, often at first glance resembled anti-C. They reacted predominantly by enzyme techniques but occasionally also gave weak positive results by IAT. As they usually gave negative results with both R_2R_2 and R_2*R_o red cells, initially their specificity was thought to be anti-hr^B. Moreover, this implied that anti-hr^B could exist alone, as well as together with anti-Rh34 [2]. Successive titrations showed, however, as the weaker examples of anti-Rh34 increased in titre, that they agglutinated red cells with weak Rh34 antigen more readily. The apparent anti-C were later shown to have anti-Ce and later still anti-Rh34 specificity.

When the stronger examples of anti-Rh34 were titrated, their titres were almost always found to be one or two doubling dilutions lower with R_2*R_o and $*r'^sR_2$ than with R_2R_2 red cells. Subsequent absorption-elution studies showed that the eluates from the R_2*R_o and $*r'^sR_2$ red cells had anti-Rh34 specificity. Although the auto-control tests were usually negative, a very few were barely, perhaps even falsely, positive microscopically. As auto-elution studies were not made, these few positive results may show that some anti-Rh34 have Rh34 auto- or Rh34 variant allo-antibody specificity. When the stronger examples of anti-Rh34 were absorbed with Rh:–34 red cells, Rh34 antibodies were not identified in the eluates.

Multiple Antibody Specificities

The presence of anti-D in addition to anti-Rh34 was suspected when the sera agglutinated D+ panel cells by IAT (table 1). This was confirmed when the sera were subsequently found to agglutinate D– – but not Rh_{null} red cells. When tested with D mosaic red cells (some typed with Wiener's original sera and others, in Durban and London, with antibodies specific for D category red cells), the anti-D from the D+ persons was found to have anti-Rh^D specificity. This explained why sera containing antibodies preliminarily identified as anti-Rh34 (for urgent transfusion purposes) sometimes gave positive results with some but not all the $*R_o$ Rh:–34 units cross-matched: undetected anti-Rh^D was present as well; while the incompatible units had normal D, the compatible red cells had the Rh^d (D category III) antigen. During this study, 2 people with $*R_o^d$ red cells proved useful sources of anti-Rh^D.

Anti-E in addition to anti-Rh34 was suspected when the sera gave higher titres than expected with R_2R_2 red cells. This was difficult to confirm, however, for anti-Rh34 and anti-E were almost always recovered in the eluates when the sera were absorbed with R_2R_2 or with r″r″) red cells. Since absorptions with R_1R_1 red cells were not made, and absorptions with homozygous –DE and – –E red cells were not possible as these phenotypes have not yet been recorded, separable anti-E was only surmised.

Anti-C in addition to anti-Rh34 was identified when the sera agglutinated both r′r and $*r'^s$ (genetically $*dCce^s$/$*dCce^s$, see later) red cells. Anti-V in addition to anti-Rh34 was identified when the sera agglutinated V+ $*R_o$ but not V– $*R_o$ red cells.

Two sera containing anti-Rh34 were found to react as weakly with R_o Rh32 (B.B.) and known homozygous Rh32 (Weibel) as with R_2R_2 red cells. However, Ann Bastiaan's unabsorbed serun gave stronger positive results with the two Rh32 samples. The findings suggested that Ann Bas-

Table 3. Maternal antibody specificity and cord blood DAT results recorded in 16 pregnancies

Maternal antibodies	Direct antiglobulin test on cord blood cells		Total
	positive	negative	
Anti-Rh34	4	6	10
Anti-Rh34, -C	0	1	1
Anti-Rh34, -D	2	0	2
Anti-Rh34, -D, -Jk^b	1	0	1
Anti-D	1	0	1
Anti-E	1	0	1
Total	9	7	16

Same cases as in table 1 for which cord blood was available. None of the infants required an exchange transfusion (see text).

tiaan's serum contained either additional anti-Rh32 or additional anti-Rh^D which had reacted with their enhanced D antigen. The anti-Rh34 gave strong positive results with a sample of r^G red cells (Fontaine).

Anti-Rh34 in Pregnancy

Table 3 lists the antibodies identified in 16 multiparae, about half of whom had received blood transfusions. The median number of pregnancies per mother was 3.5, and the number of children per mother ranged from 1 to 10. The cases were all those in table 1 from whom cord blood had been received for testing. Among the 10 mothers with anti-Rh34 only, 4 gave birth to infants whose cord cells were direct antiglobulin test (DAT)-positive. The antibodies in 3 of the 4 mothers reacted by saline, one- and two-stage enzyme and indirect antiglobulin techniques (IAT). The titres were 64–256 by two-stage enzyme technique and 16 by IAT. The anti-Rh34 in the 4th mother gave negative results by saline technique and reacted weakly by one- and two-stage enzyme techniques and by IAT. None of the infants suffered more than mildly from haemolytic disease of the newborn (HDN), and no exchange transfusions were required.

The 4 multiparae in whom anti-Rh34 and anti-D or anti-D only were identified also gave birth to infants whose cord cells were DAT positive. Their anti-D was specific for the Rh^D portion of the D antigen mosaic. Their anti-Rh34 titres were 8–16 by one- and two-stage enzyme techniques, but negative or weakly positive results were obtained by IAT. The infants were not typed for D or Rh34, and eluates were not made from their cord cells. None of the infants needed treatment for HDN.

Six multiparae with anti-Rh34 and 1 with anti-Rh34 and anti-C gave birth to infants whose cord cells were DAT negative. The anti-Rh34 of 2 reacted by saline, one- and two-stage enzyme techniques and by IAT. The anti-Rh34 of 2 others reacted by saline and by one- and two-strage enzyme techniques only. In the remaining 2 multiparae, the anti-Rh34 reacted by one- and two-stage enzyme techniques and by IAT. The titres by two-stage enzyme technique were 32 or less. No neonatal details for the anti-E case were available.

Rh:–34 Homozygous and Heterozygous Red Cells

Table 4 contains the Rh phenotypes and probable Rh genotypes identified in 49 antenatal patients and transfusion recipients with Rh:–34 red cells. The D-negative Rh:–34 red cells gave negative results with Ann Bastiaan's unabsorbed serum and with other unabsorbed sera known to contain anti-Rh34. The D-positive Rh:–34 red cells gave negative results with many examples of unabsorbed anti-Rh34 and 2 selected strong examples from which anti-D and other common antibodies were known to have been excluded. Twenty-eight of the subjects had anti-Rh34 only or anti-Rh34 plus other antibodies in their sera. The Rh phenotypes and probable genotypes of 42 Rh:–34 blood donors and of 9 Rh:–34 parents engaged in disputed paternity or maternity tests are also shown. The blood donors were found while screening for crossmatch-compatible blood, and the parents when the paternity tests revealed exclusions in the Rh system which were not confirmed by exclusions in other systems. The Rh phenotypes and probable genotypes of 61 Rh34 heterozygotes, 2 of whom had made anti-C-like Rh34 antibodies, are given in this table as well. Their results were determined from 22 Rh:–34 family and mother-child studies. All the blood samples gave negative DATs with polyspecific antiglobulin reagents. From the findings, 4 haplotypes not encoding Rh34 were identified. They were $*r''$ *(*dCce)*, $*R^1$ *(*Dce)*, $*R^{o_m}$ *(*D"ce)* and $*R^{o_d}$ (category III **Dce*). The haplotypes R^2 *(DcE)* and r'' *(dcE)* encoded weak Rh34 antigen, the latter a trifle more than the former. Titrations with anti-Rh34 confirmed that less Rh34 antigen was produced by R^2/R^{-34} than R^2/R^2 genotypes. The partially deleted $\bar{R}^o$ *(Dc–)* haplotype identified will not be discusse further here. Anti-Rh34-compatible r''r red cells, their phenotype suggesting r''^s/r *(dCce^s/dce)* genes, were found instead to be the products of homozygous $*r''^s/*r''^s$ *(*dCce^s/*dCce^s)* genes.

The haplotype $*R^{o_m}$ *(*D"ce)* was identified in *trans* to $*r''^s$ *(*dCce^s)* in 2 families and 6 of 100 unrelated subjects. No homozygotes were found. Nineteen subjects with $*r''^sR_o$ red cells confirmed that their D^u was not a depressed D antigen

Table 4. Phenotypes and probable genotypes of Rh:–34 propositi and their family members and those of other individuals with heterozygous R^{-34} haplotypes

Phenotype	Probable genotype	Antenatal patients	Crossmatch patients	Blood donors	Paternity cases	Total
$*R_o$	$*R^o/*R^o$	10	7	17	5	39
$*R_o^u$	$*R^{ou}/*R^{ou}$	3	3	0	0	6
$*R_oR_o$	$*R^o/R^o$	1	0	0	0	1
$*r'^s/R_o$	$*r'^s/R^o$	3	2	0	0	5
$*r'^sR_o$	$*r'^s/*R^o$	8	4	5	2	19
$*r'^sR_o^u$	$*r'^s/*R^{ou}$	3	2	3	1	9
$*r'^sr$	$*r'^s/*r'^s$	1	2	17	1	21
R_o	$R^o/*R^o$	5	3	2	2	12
R_1r	$R^1/*R^o$	0	1	0	1	2
R_1r	$R^{1u}/*R^o$	2	0	0	0	2
r'^sR_o	$*r'^s/R^o$	3	2	0	0	5
$r'^sR_o^u$	$*r'^s/R^{1u}$	4	0	0	0	4
r'^sR_2	$*r'^s/R^2$	3	0	5	2	10
r'^sr'	$*r'^s/r'$	1	0	0	0	1
r'^sr''	$*r'^s/r''$	0	0	1	0	1
r'^sr	$*r'^s/r$	0	1	0	1	2
R_2R_o	$R^2/*R^o$	3	3	13	2	21
R_2R_o	$R^2/*R^{ou}$	0	0	1	0	1
Total		50	30	64	17	161

To conserve space, only the Rh shorthand symbols are shown. The genotypes were determined from 22 family and mother-child studies; the Rh:–34 phenotypes and haplotypes are indicated by * signs.

Table 5. Results showing that Rh^d (D category III) was the most frequent D mosaic antigen identified in D+ Rh:–34 red cells

D Mosaic antigen	$*R_o$	$*R_o^u$	$*r'^sR_o$	$*r'^sR_o^u$
Rh^{ABCd}	1	0	1	1
$Rh^{AB.d}$	3	1	0	1
$Rh^{A..d}$	1	0	1	2
$Rh^{...d}$	2	1	0	0
$Rh^{Ab.d}$	0	0	0	1
Rh^{*BCd}	0	0	0	1
'variant'	2	0	2	0
Total	9	2	4	6

Dots indicate where insufficient reagent was available to complete the test.

due to the C+ haplotype in *trans* [8]. When the patients' serum contained antibodies to both Rh34 and D, often $*r'^sR_o^u$ and $*R_o^u$ were the only blood units compatible.

Table 5 shows the results when 9 $*R_o$, 2 $*R_o^u$, 4 $*r'^sR_o$ and 6 $*r'^sR_o^u$ red cells samples were tested with Wiener's antibodies specific for the Rh^A, Rh^B, Rh^C and Rh^D portions of the D antigen mosaic. Dots indicate where insufficient reagent was available to complete the study. The phenotype which occurred most commonly was Rh^d (D category III) [9, 10].

Eighteen $*R_o$, 3 R_o^u and 21 $*r'^sr$ *(*dCce^s/*dCce^s)* red cell samples gave positive results with anti-G. Negative results with anti-Go^a, -D^w, -Rh32 and -Rh33 confirmed that the corresponding antigens were not present. Negative results in saline tests with incomplete anti-D reagents also confirmed that the $*R_o$ red cells lacked enhanced D antigen.

Family Studies

Figure 1 shows the W.V. and E.J. pedigrees. The Rh:–34 haplotypes in the W.V. family were *$*r'^s$ (*dCce^s)* and *$*R^o$ (*Dce)*, and those in the E.J. family *$*R^o$ (*Dce)*, *$*r'^s$ (*dCce^s)* and *$*R^{ou}$ (*D^{u}ce)*. The Rh inheritance in both families was in accordance with the normal Mendelian dominant pattern. The mother in the W.V. family had made anti-Rh34 and the mother in the E.J. family anti-Rh^D.

Frequency Studies

The frequency of $*R_o$, $*R_o^u$ and $*R_o^d$ phenotypes in D-positive Black and Coloured blood donors was estimated to be 0.27% (7 in 2,631). In D-C+ Black and Coloured

blood donors, however, the frequency of *r''r *(*dCce^s/*dCce^s)* phenotypes was 4.08% (4 in 98). Although some of the D-C+ donors may have donated more than once during the study period, the higher Rh:–34 frequency in them showed that they are the best individuals to screen when this phenotype is required urgently for transfusions.

Since the *r'' *(*dCcde^s)* haplotype was known to encode VS antigen [11], a serum containing anti-VS was used to estimate the VS, and therefore the *r''r *(*dCce^s/*dCce^s)*, phenotype frequency in a selected sample of 153 random blood donors with group 0 r'r red cells. All of the 73 red cells samples from Blacks and 3 of the 12 from Coloureds, but none of the 43 from Whites or of the 22 from Asians, gave positive results. The findings confirmed that *r''r *(*dCce^s/*dCce^s)* red cells occurred predominantly in Blacks.

Paternity Testing

Figure 2 explains how anti-Rh34 gave the correct diagnosis in a disputed paternity test. If this reagent had not been included among those used for phenotyping, the putative father would not have been excluded. His R_2r phenotype suggested that he had *R^2r (DcE/dce)* genes, and the r'r phenotype of the child's red cells suggested that her genes were *r'/r (dCe/dce)*. When anti-Rh34 was included, the putative father was acquitted, for the child's *r''/*r'' Rh:–34 phenotype implied that her genotype was *r''/*r'' *(*dCce^s/*dCce^s)*. Comparative titrations also showed that Rh34 antibodies distinguished, both by titre and score, between heterozygous and homozygous Rh34 antigen, i.e. they detected *RH 34* dosage.

Haematological Findings

Detailed blood examinations in 4 persons with Rh:–34 red cells showed no evidence of any haemotological abnormalities.

Discussion

In this study, Rh34 antibodies were found to be specific for a high-frequency Rh antigen which had not so far been adequately characterised. No evidence that the antibodies had the vague anti-Hr-like specificity suggested by Shapiro et al. [2] was detected. Moreover, partially deleted and Rh_{null} phenotypes were not the only red cells compatible. When sera containing anti-Rh34 were incompletely absorbed with R_2R_2 red cells, their reactivity was merely reduced: the absorbed sera no longer agglutinated R_2R_2 red cells or red cells with weak Rh34 antigen. Absorption-elution studies also revealed that the absorbed sera, and other sera

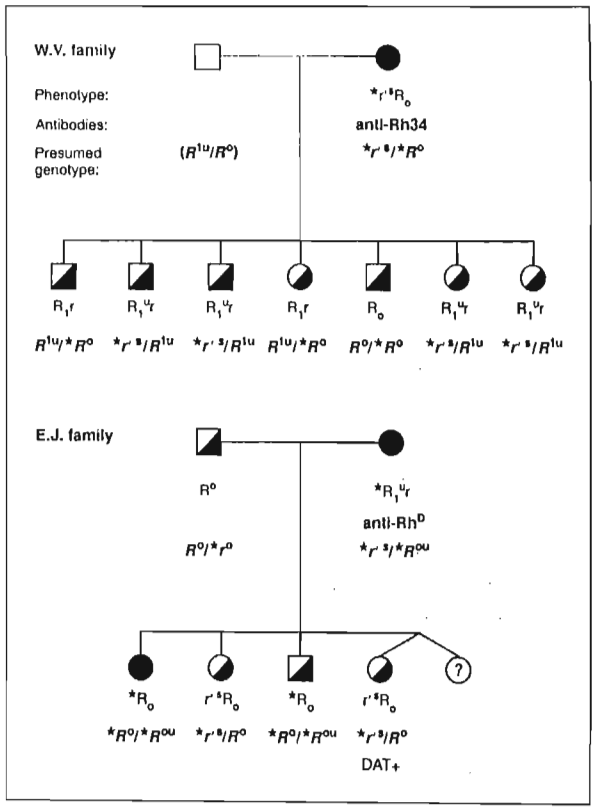

Fig. 1. The W.V. family in which *r'' *(*dCce^s)* and *R'' *(*Dce)*, and the E.J. family in which *r'' *(*dCce^s)*, *R'' *(*Dce)* and *R''' *(*D''ce)* haplotypes were present. The closed circles represent the members with *Rh:–34 phenotypes and haplotypes; the half-closed circles and squares represent the heterozygotes; open circle and square indicate the members whose red cells were not tested.

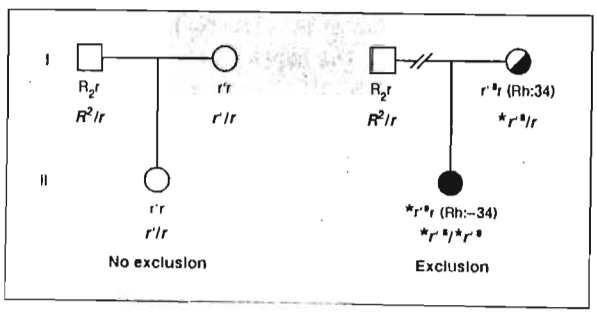

Fig. 2. This figure explains how a man wrongly accused in a disputed paternity case was acquitted when Rh34 antibodies were included among the reagents used to phenotype the red cells. On the left, the open circles and squares indicate that anti-Rh34 was not used. On the right, the closed circle shows that the child had an *Rh:–34 phenotype and haplotypes. The half-closed circle indicates that the mother was heterozygous, and the open square shows that the accused man had an Rh:–34 phenotype and haplotypes.

thought to contain hr^B antibodies (because they gave positive results with all except R_2R_2, D-deletion and Rh_{null} red cells), in reality contained Rh34 antibodies. Shapiro et al. [2] used the term anti-hr^B for the antibodies remaining in Mrs. Bastiaan's serum after her serum had been partially absorbed with R_2R_2 red cells.

The studies showed that anti-e made by R_2R_2 subjects was unlikely to be anti-Rh34 or a mixture of anti-e and anti-Rh34 because R_2R_2 red cells carried weak Rh34 antigen. For the reasons given in the previous paragraph, neither would the anti-e be likely to have anti-hr^B or anti-e+hr^B specificity. The term anti-hr^B merely implied that a serum containing anti-Rh34 had been partially absorbed with R_2R_2 red cells.

Sera which agglutinate all except partially deleted, Rh_{null} and R_2R_2 red cells, and agglutinate C+ more strongly than C– red cells, are customarily believed to contain hr^B antibodies. Shapiro et al. [2] emphasised that anti-hr^B was e-like and compatible with R_2R_2 red cells. As a result, patients with Rh34 antibodies may be unwisely yet confidently transfused with units of R_2R_2 blood. Provided that anti-hr^B is recognised as being an antibody unlikely to occur on its own, shortened red cell survival times and minor transfusion reactions can be explained by postulating the presence of anti-Rh34.

The present study showed that some red cells with common e-positive phenotypes, in addition to partially deleted and Rh_{null} red cells, were compatible with Rh34 antibodies. In family and mother-child studies, the e-positive phenotypes were found to be due to the inheritance of a pair of, or any 2 of, the haplotypes *r''^s *(*dCce^s)*, *R^o *(*Dce)*, *R^{on} *(*D^{u}ce)* and *R^{od} (category III **Dce)*. An alternative term for *r''^s was r''^n [12, 13]. In 1960, Zoutendyk and Teodorczuk [14] recorded that r''^n occurred relatively frequently in the South African Blacks. The haplotypes *r''^s *(*dCce^s)*, *R^o *(*Dce)* and *R^{od} (category III **Dce)* correspond to 3 of the 5 which Shapiro et al. [2] had said determined hr^B-negative red cells. Their 4th and 5th haplotypes were r and $\bar{R}^2$. In our study, neither a *r *(*dce)* haplotype nor *R^1 *(*DCe)*, *R^z *(*DCE)*, *r' *(*dCe)* or *r^y *(*dCE)* haplotypes were identified. The R^2 *(DcE)* haplotype was known to encode weak Rh34 antigen. Different red cell samples typed either as hr^B-negative Rh:–34, hr^B-negative Rh:34 and hr^B-positive Rh:34; none typed as hr^B-positive Rh:–34. The D–C+E–c+e+ phenotype associated with both *r''r and r'r, and the D+C–E–c+e+ phenotype associated with both *R_or and R_or, provided further proof that Rh34 belongs to the Rh system.

The Rh^d Rh:–34 were found to outnumber the Rh^D Rh:–34 red cell samples in this study by almost 2:1. Since Issitt [15] believes that unusual Rh phenotypes are more often than not accompanied by multiple anomalous antigens, the situations which give rise to Rh^d and Rh:–34 red cells may be related in some way. Transfusions of *R_o blood evidently stimulated anti-Rh^D in recipients who had *R_o^d red cells. Not all the units of D-positive Rh:–34 blood which were subsequently crossmatched for them proved compatible; the incompatible units were *R_o and the compatible units *R_o^d (D category III). The most useful units of blood were therefore *r''^{s}R_o^u, *R_o^u and *r''^{s}.

Weak, predominantly enzyme-reacting Rh34 antibodies which resembled anti-C were frequently identified in subjects who had R_2*R_o or *r''^{s}R_2 red cells. Their red cells were found to express less Rh34 antigen than R_2R_2 red cells. The anti-Rh34 in their sera may have been auto-antibody. Alternatively, their R^2 *(DcE)* haplotypes encoded subtype Rh34 antigen, and their sera contained Rh34 variant antibodies. A suggestion that the antibodies in these subjects had anti-hr^B specificity due to auto-absorption in vivo was not considered realistic.

The majority of the examples of anti-Rh34 identified in this study reacted optimally by enzyme techniques. Although those with the highest enzyme titres also gave positive results in saline and by IAT, only mild HDN was recorded, and no exchange transfusions were needed. When exchange transfusions were required, the maternal serum was usually found to contain other antibodies, especially Rh, in addition to anti-Rh34.

Antigen dosage titrations with anti-Rh34 showed that these antibodies could be used to decide whether or not the alleged parents and children in disputed maternity or paternity cases had R^{-34} in trans to common Rh haplotypes. For example: when the subject had an apparent r'r phenotype and the control a known *r'/r* genotype, high titres and scores suggested that the subject's genotype was *r'/r (dCe/dce)*. Lower titres and scores suggested that it was *r''/r (*dCce^s/dce)* and negative results that it was *r''^{s}/r''^{s} (*dCce^s/dCce^s)*. Similarly, when the subject had R_o red cells and the control a known R^o/R^o *(Dce/Dce)* genotype, high titres and scores suggested that the subject's genotype was R^o/R^o *(Dce/Dce)*. Lower titres and scores suggested that it was R^o/*R^o *(Dce/*Dce)* and negative results that it was *R^o/*R^o *(*Dce/*Dce)*.

The serological resemblance between the phenotypes *r''^{s}r *(*dCce^s/*dCce^s)* and r'r *(dCe/dce)*, and between the phenotypes *R^o *(*Dce/*Dce)* and R^o *(Dce/Dce)*, suggested (1) that *r''^s *(*dCce^s)* was a mutation of *r' (dCe)* and *R^o *(*Dce)* a mutation of R^o *(Dce)*; (2) that *R^{on} *(*D^{u}ce)* and *R^{od} (category III **Dce)* were later mutations of *R^o *(*Dce)*, and (3) that the membranes of Rh:–34 red cells

contained one or more defective Rh-related polypeptides that prevented production of Rh34 antigen. Issitt [15] speculates that Rh gene DNA comprises a series of exons and introns, such that different transcription units, with different control mechanisms and/or splicing instructions, may give rise to related but slightly different proteins. Rh34 antibodies may therefore be stimulated when the membranes of the donor's red cells contain normal, i.e. Rh:34, while those of the recipient's red cells contain defective, i.e. Rh:–34, Rh-related polypeptides.

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Dr. P. Moores
Immuno-Haematology Research Laboratory
Natal Institute of Immunology
PO Box 2356
4001 Durban (South Africa)

STEM, A NEW LOW FREQUENCY ANTIGEN LINKED TO Rh AND ASSOCIATED WITH THE RED BLOOD CELL PHENOTYPES hr^{S}- (Rh:-18,-19) AND hr^{B}- (Rh:-31,-34)

I. Marais*, P. Moores+, E. Smart+ and R. Martell*.

*Provincial Laboratory for Tissue Immunology, Cape Town, +Natal Institute of Immunology and +Natal Blood Transfusion Service, Durban, South Africa.

Running title: STEM, a new low frequency antigen

Correspondence:

Dr Phyllis Moores, Head, Immuno-haematology Research Laboratory,
Natal Institute of Immunology, P.O. Box 2356, 4000 Durban, South Africa.

SUMMARY. The "new" low incidence antigen STEM was identified when Mrs F.S., a Cape Coloured woman with an unknown antibody in her plasma which agglutinated her husband's red cells and those of a few other individuals, gave birth to a baby suffering mildly from haemolytic disease of the newborn. Most, but not all, examples of anti-STEM distinguished different strengths of STEM on the red cells of different people; the different strengths were inherited. Three family studies established that the gene determining STEM was a Mendelian dominant character. The total lod score of (to be determined) at a recombination fraction of 0.0 indicated that STEM was closely linked to the Rh system. STEM was also closely associated (in linkage disequilibrium) with the phenotypes hr^{S}- (Rh:-18,-19) and hr^{B}- (Rh:-31,-34), subdividing each into STEM+ and STEM-. Further examples of anti-STEM have been found. STEM has been provisionally allocated the ISBT Rh number 49.

Key words: Human blood groups; Rh system; Low frequency antigen; e variant phenotypes; hr^{S} and hr^{B}.

The hr^s- (Rh:-19) phenotype was first described by Shapiro in 1960. Anti-hr^s (-Rh19) was the e-like antibody which remained in the serum after anti-Rh18, an antibody to a very high frequency antigen that the author said was compatible only with red cells having "deleted" and Rh_{null} phenotypes, had been partially absorbed with R_2R_2 red cells. The hr^B- (Rh:-31) phenotype was first described by Shapiro, Le Roux and Brink in 1972. Anti-hr^B (-Rh31) was the e-like antibody which remained in the serum after anti-Rh34, an antibody to another very high frequency antigen which these authors said was compatible only with red cells having "deleted" and Rh_{null} phenotypes, had also been partially absorbed with R_2R_2 red cells. In contrast, Rh:-34 red cells with otherwise common Rh phenotypes (R_o, R_o^u, R_1r, R_2r) were reported by Moores and Smart in 1991.

In this paper, a new low frequency blood group antigen which we have named STEM and which has been provisionally allocated ISBT Rh number 49, is described. STEM is closely linked to the Rh system and is associated with the phenotypes hr^s- (Rh:-18,-19) and hr^B- (Rh:-31, -34), each of which it subdivides into STEM+ and STEM-. Some examples of anti-STEM distinguish different strengths of STEM antigen on the red cells of different individuals. Anti-STEM is an IgG antibody, but it has so far caused only mild haemolytic disease of the newborn.

MATERIALS AND METHODS

The blood samples were tested within 48 hours of venesection. Aliquots of the red cells were also stored in glycerol at -32°C and recovered by dialysis against saline when required. Standard blood grouping techniques were used (Widmann, 1981). The enzymes employed were bromelin and ficin; the antiglobulin reagent was polyspecific anti-IgG plus anti-complement. The eluates were made using Elu-Kit II (Gamma Biologicals), heat, ether and digitonin rapid acid-stromal elution techniques. The sensitized red cells were de-sensitized using Gamma-Quin, a chloroquine diphosphate solution (Gamma Biologicals). In the linkage analysis, the lod scores were kindly calculated by Professor M. Lewis (Rh Laboratory, Winnipeg, Canada) from seven

family studies; the recombination fractions for males and females were assumed to be equal.

CASE HISTORY

Mrs F.S., who had two children (not tested) by a previous husband, was first seen in 1980 when her third baby's cord blood cells gave a 1+ (weakly positive) result in a direct antiglobulin test (DAT). Mrs F.S. was group A_2B, R_1r, Jk(a-b+), and her baby's red cells were typed as A, Rh+, Jk(a+b+). The baby subsequently became mildly jaundiced, but was successfully treated with phototherapy. Serological tests for syphilis were negative. Mrs F.S.'s serum reacted 2+ (moderately positive result) by indirect antiglobulin technique (IAT) with her baby's red cells and one sample in a commercial antibody identification panel (Ortho). The panel sample was group O, R_o, hr^s-, Jk(a+b-) [personal communication to P. Moores by B. Sabo, Ortho Research Foundation, New York, USA]. Mrs F.S. received four units of compatible blood to correct her anaemia.

In 1983, Mrs F.S. gave birth to her fourth baby. The baby's cord cells reacted 2+ by DAT. Anti-E, anti-Jk^a and the previously detected antibody for an unknown low frequency antigen were identified in her serum. In titrations by bromelin technique, the unknown antibody agglutinated to titre 8-64 her husband's and other E+ red cells, but in titrations by IAT it agglutinated only her husband's red cells (titre 4). The husband was group O, R_2r, Jk(a+b-) and the baby group A, R_1R_2, Jk(a+b+). In the eluate prepared from the baby's cord cells by the 56°C heat elution technique of Landsteiner and Miller (1925), anti-E and anti-Jk^a only were detected.

Mrs F.S.'s fifth baby was born in 1990. During the antenatal period, only her unknown antibody was detected. At 37 weeks, titrations showed that it reacted to titre 1 by enzyme and to titre 2 by IAT. The baby was group A, R_1r, and the cord cell DAT was positive. Following chloroquine treatment, the cord cells typed as Jk(a+b+). The unknown antibody was successfully eluted from the cord cells using Elu-Kit II. The baby became

mildly jaundiced, but was successfully treated with phototherapy.

SEROLOGICAL RESULTS

Characterization of anti-STEM

The unknown antibody in Mrs F.S.'s serum was given the name "anti-STEM" (part of the family surname). After the birth of her fourth baby, a sample of her serum was absorbed three times with R_2R_2, Jk(a+b-) red cells, following which no trace of her anti-E or anti-Jk^a was detected. The absorbed serum reacted with STEM+ red cells optimally by the one- and two-stage bromelin techniques at 20-22°C after gentle centrifugation. Some red cell samples gave strong positive (3+ to 4+), others weak to moderate positive (1+ to 2+) and a few others microscopic positive results. By a one-stage ficin tile technique at 20-22°C and by IAT, weaker results were obtained. With untreated red cells at 20-22°C and at 37°C, all the results were negative.

In titrations with strongly STEM+ red cells, Mrs F.S.'s serum (post fifth baby) gave titres of ±8 by one-stage bromelin, 16-32 by two-stage bromelin and 4-8 by IAT. One absorption with strongly STEM+ red cells reduced the two-stage bromelin titre from 16 to 4, and two absorptions removed all trace of anti-STEM. Eluates were successfully obtained by the heat and digitonin rapid acid-stromal but not by the ether elution technique.

Mrs F.S.'s anti-STEM was not inhibited by ABH secretor or non-secretor saliva, pigeon egg white, human milk or Guineapig urine. It was not inactivated by dithiothreitol (DTT), but agglutinated strongly STEM+ red cells treated with either 2-aminoethylisothiouronium bromide (AET) or with brom-ZZAP (bromelin substituted for papain) solution.

Further examples of antibody

Further examples of anti-STEM have since been found, in post-

natal sera from mothers whose newborn babies' red cells unexpectedly gave positive DAT results. Most examples reacted best by enzyme techniques, but one gave stronger positive results by IAT and failed to distinguish between the different grades of STEM antigen. In the latter case, although mildly affected, the baby recovered uneventfully.

Linkage analysis and family studies

The results of the linkage analysis are shown in Table 1. The total lod score of (being recalculated) at a recombination fraction of 0.0 provided good evidence that STEM was closely linked to Rh.

Mrs F.S. (I-2, Fig. 1) and her family gave the first indication that STEM was closely associated with a particular Rh haplotype, namely $\hat{R}^{o}$. I-3, whose red cells were hr^{s}- and strongly STEM+, evidently had an $R^{2}/\hat{R}^{o}$ genotype. He had given his $\hat{R}^{o}$ haplotype with STEM to II-3 and II-5 and his R^{2} haplotype without STEM to II-4. The R^{1} haplotypes in II-3, II-4 and II-5 must have been inherited from I-2. The mother of I-3 was deceased and his father could not be traced. As II-3 and II-4 were V-, they had not inherited their mother's R^{ov} haplotype.

In Fig. 2, II-1 and II-7 evidently had $R^{2}/\hat{R}^{o}$, and II-4 an $R^{1}/\hat{R}^{o}$, genotypes and their red cells had strongly expressed STEM antigen. As I-2, II-2, II-6 and II-8 had no R^{o} haplotype and had STEM- red cells, in this branch of the family the gene determining STEM was clearly segregating with $\hat{R}^{o}$. In the other branch of this family, I-4, II-10 and II-12 had weakly STEM+ red cells. The R_{o} hr^{B}- (Rh:-31,-34) red cells of II-10 and II-12 and the STEM- red cells of I-3 showed that the gene encoding weak STEM expression was segregating with their maternal $\hat{R}^{o}$ haplotype.

The family in Fig. 3 revealed that the gene encoding strong STEM expression was dominant to the gene for the weakly expressed form. The family also confirmed that both forms of STEM were inherited. The genotype of I-2, who had STEM- red

cells, might be either R^{o}/r or R^{o}/R^{ou}. For simplicity, the former was assumed present. The genotype of I-1, who had R_o hr^s- red cells with strongly expressed STEM antigen, was evidently either $\hat{R}^{ou}/\hat{R}^{ou}$ or $\hat{R}^{ou}/\hat{r}$. The former was again assumed present, as the existence of an $\hat{r}$ (Rh:-18,-19) haplotype (Shapiro, 1960) was questionable (P.Moores, unpublished results). I-1 had apparently given an $\hat{R}^{ou}$ haplotype with strong STEM expression to II-3, II-4, II-5, II-6 and II-7, and an $\hat{R}^{ou}$ haplotype with weak STEM expression to II-2. The genotypes of III-1, III-2 and III-3 showed that II-1, who surprisingly also had R_o hr^s- red cells and a strongly STEM+ phenotype, also had an $\hat{R}^{ou}/\hat{R}^{ou}$ genotype! Evidently, II-1 had given an $\hat{R}^{ou}$ haplotype encoding weak STEM to III-1 and III-3 and an $\hat{R}^{ou}$ haplotype encoding strong STEM to III-2. II-2 must have provided III-1 and III-3 with an ordinary R^{o} haplotype and III-2 with an $\hat{R}^{ou}$ haplotype encoding weak STEM antigen.

Non-identity with other antigens

The following antigens and phenotypes were excluded from being the same as STEM in tests using either Mrs F.S.'s serum or another example of anti-STEM, or by typing the red cells of Mrs F.S.'s husband or those of other individuals having strong STEM antigen with the corresponding antibodies: An^a, Be^a, Bg^a, Bg^c, Bi^a, Bu^a, Bx^a, By^a, C^w, C^x, Cl^a, Co^b, Dantu, Dh^a, Di^a, E^w, Evans, Fr^a, Go^a, He+, Hey, Hg^a, Hut, In^a, Je^a, JFV, Js^a, K, Kn^b, Kp^a, Kp^c, Li^a, Ls^a, Lu^a, Lu9, Lu14, M^g, M^v, Mi^a, Milne, Mit, Mi.I, Mi.II, Mi.III, Anek, Raddon, Mo^a, Mt^a, Mur, Ne^a, NFLD, Ny^a, Or, Pt^a, Rb^a, Rd, Re^a, Rh23, Rh32, Rh33, Rh35, Rh40, Rh42, Rh43, Ri^a, s^b, St^a, Sw^a, Sw1, Tc^c, Tr^a, TSEN, Ul^a, V, VS, Vg^a, V^w, Wb, Wd^a, Wk^a, Wr^a, Wu, Xg^a, Yt^b and 20 other unpublished low frequency red cell antigens.

Red cells lacking high frequency antigens of the following phenotypes which were not agglutinated by anti-STEM were: Co(a-b-), Fy(a-b-), Gn(a-), Hy-, I-, Jk(a-b-), Jo(a-), Jr(a-), Js(a+b-), Kn(a-), K_o, Lu(a-b-), LW(a-b-), McC(a-b-), O_h, Ok(a-), PP_1P^k-, Rg(a-), Rh_{null}, S-s-U-, Tc(a-b-c+), Vel- and Yt(a-).

Population frequency studies

The distribution of STEM+ red cells in the Natal (Zulu) and Cape (Xhosa) Blacks, and in the Coloured (mixed race), White and Indian populations of both the Cape and Natal, was estimated using the sample of anti-STEM that reacted preferentially by IAT. In titrations, the sample gave titres of 16 with strongly and 4 with weakly STEM+ red cells. Table 2 shows that the highest STEM frequency occurred in the Blacks and Coloureds. Using Mrs F.S.'s anti-STEM diluted 1/2 in enzyme tests, Lewis and Kaita (personal communication to P.Moores) failed to identify STEM+ red cells in 206 Whites or 71 Indians living in Canada.

Association with phenotypes hr^S- and hr^B-

Table 3 contains the number and frequency of subjects with STEM+ and STEM-, hr^S- and hr^S+, and hr^B- and hr^B+, red cells found in Natal and Cape Black and Coloured subjects combined. Approximately 65% of the hr^S-, and approximately 30% of the hr^B-, were STEM+. The hr^S- STEM+ outnumbered the hr^B- STEM+ subjects by 5.5 to 1. The χ^2 calculations for probability of association under-estimated the STEM+ hr^S- and STEM+ hr^B- subjects substantially as, although STEM was the product of a dominant gene, hr^S- and hr^B- were due to the presence of homozygous recessive genes.

In 62 Natal Black and Coloured trios, comprising mothers, children and putative fathers, two mothers were found to have STEM+ red cells. One mother had an R_2r hr^S- phenotype; her genotype was expected to be $R^2/\hat{R}^\circ$, and her child's R_2R_2 STEM- red cells agreed with this. The other mother and her child had R_o phenotypes. Their expected genotypes were either $R^\circ/\hat{R}^\circ$ or $R^\circ/\hat{\bullet}^\circ$, and their hr^S+, hr^B+ (Rh:34) red cells suggested that the haplotype encoding STEM was masked by their ordinary R° haplotype *in trans*.

DISCUSSION

This study describes a new low frequency red cell antigen which we have called STEM and which has been provisionally allocated the ISBT Rh number 49. The lod scores provided good evidence that STEM was part of, or closely linked to, the Rh system; moreover, there was no indication in the families that any definite recombinants had occurred between the Rh and STEM loci. The family studies indicated that the gene responsible for STEM was inherited as a Mendelian dominant character. The serological results with one example of anti-STEM, that reacted preferentially by enzyme techniques, suggested that STEM antigen varied in strength; most but not all STEM antibodies recognised this quantitative variation. The family studies showed that the strength of STEM was not related to either homo- or heterozygosity.

The family in Fig. 1 and the R_o hr^s- STEM+ red cells of the Ortho panel cell sample strongly suggested that STEM was associated with, or was in linkage disequilibrium with, an $\hat{R}^\circ$ haplotype. The family in Fig. 2 and other studies suggested that STEM was also associated with an $\dot{R}^\circ$ haplotype. Not all $\hat{R}^\circ$ and $\dot{R}^\circ$ haplotypes encoded STEM, however; see Table 3. In addition, individuals with STEM+ hr^s+ or STEM+ hr^B+ red cells might have either $R^\circ/\hat{R}^\circ$ or $R^\circ/\dot{R}^\circ$ haplotypes, respectively. As the STEM+ hr^s- outnumbered the STEM- hr^s- blood samples, STEM was also a useful marker antigen for the hr^s- phenotype.

Anti-STEM, which is an IgG antibody, was expected to cause transfusion reactions and haemolytic disease of the newborn. Only mild HDN has been recorded so far, however. The enzyme-reacting examples distinguished readily between strongly and weakly expressed STEM phenotypes, but some sera recognised this difference only by titration. The example of IAT-reacting anti-STEM agglutinated both strong and weak STEM+ red cells equally well.

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Table 1. Lod scores estimated using STEM as the main locus and RH as the marker locus, with recombination frequencies for males and females assumed to be equal.

	Recombination Fraction (0)						
	0.0	0.001	0.05	0.1	0.2	0.3	0.4
Pedigree 1							
Pedigree 2							
Pedigree 3							
Pedigree 4			(being recalculated)				
Pedigree 5							
Pedigree 6							
Pedigree 7							
Total:							

Table 2. Frequency of STEM in South Africans not typed for hr^s (Rh19), hr^B or Rh34.

Population	Number tested	STEM+ Number	STEM+ Percent	Gene frequency
Zulu	302	13	4.3	0.022
Xhosa	414	13	3.1	0.016
Natal Coloureds	91	4	4.4	0.022
Cape Coloureds	708	42	5.9	0.030
Indians	461	2	0.4	0.002
Whites	2957	1	0.03	0.0001

Table 3: Blacks and Coloureds from Natal and the Cape of known hr^s and hr^B phenotype tested with anti-STEM.

Number tested	Phenotype	STEM+ No.	STEM+ %	STEM- No.	STEM- %
158	hr^s-	28	(17.7)	15	(9.5)
	hr^s+	55	(34.8)	60	(38.0)
	hr^B-	5	(3.2)	12	(7.6)
	hr^B+	78	(49.4)	63	(39.8)

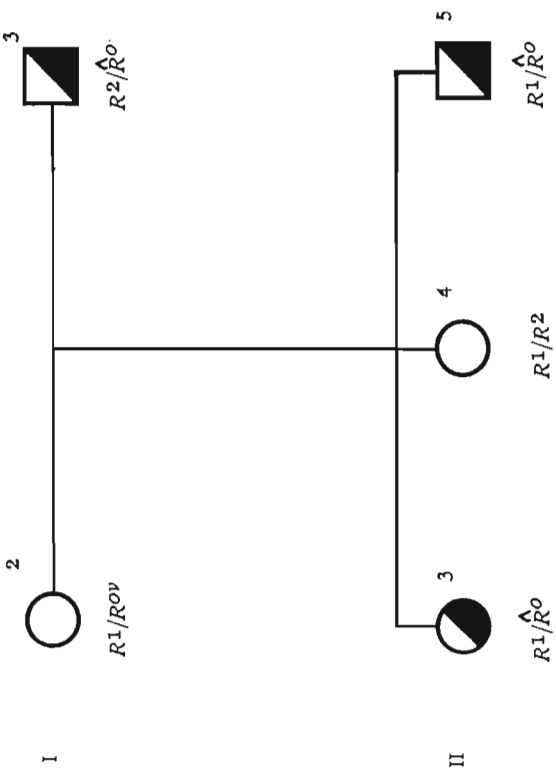
LEGENDS FOR FIGURES

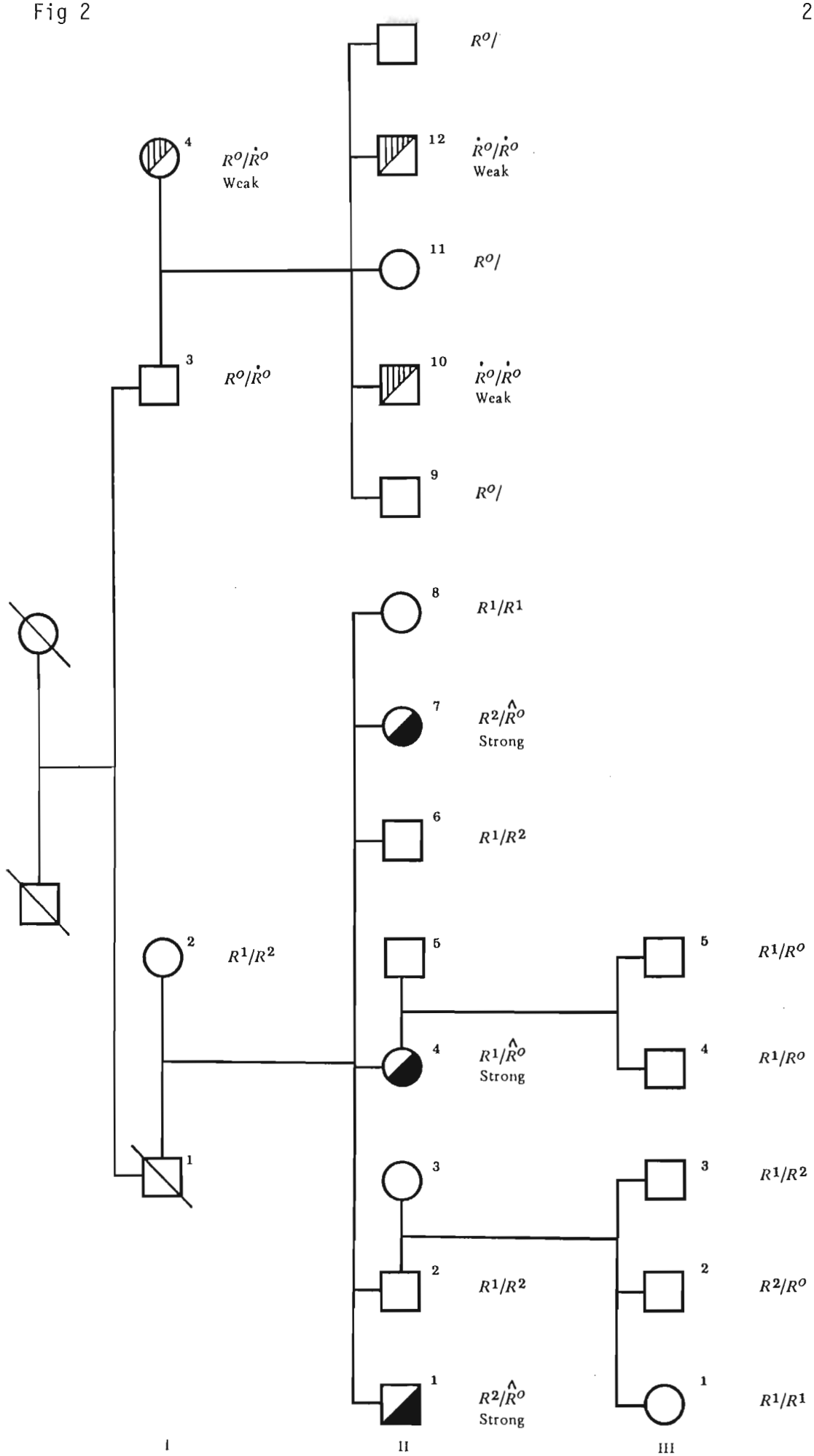
Fig. 1: Family pedigree of Mrs F.S. Not shown are her first husband, I-1, and her first two children by him, II-1 and II-2, none of whom were tested. Dark half squares and circle show STEM antigen segregating with $\hat{R}^{\circ}$.

Fig. 2: Family in which strongly expressed STEM antigen is segregating with $\hat{R}^{\circ}$ and weakly expressed STEM antigen with one of two $\dot{R}^{\circ}$ haplotypes.

Fig. 3: Family in which strongly expressed STEM antigen (dark half circles and squares) is segregating with one $\hat{R}^{\circ u}$, and weakly expressed STEM antigen (hatched half circles and squares) with another $\hat{R}^{\circ u}$ haplotype.

Fig 1




$R^o/$
12
$\dot{R}^o/\dot{R}^o$
Weak
4
$R^o/\dot{R}^o$
Weak
11
$R^o/$
3
$R^o/\dot{R}^o$
10
$\dot{R}^o/\dot{R}^o$
Weak
9
$R^o/$
8
R^1/R^1
7
$R^2/\hat{R}^o$
Strong
6
R^1/R^2
2
R^1/R^2
5
4
$R^1/\hat{R}^o$
Strong
3
2
R^1/R^2
1
$R^2/\hat{R}^o$
Strong
1
5
R^1/R^o
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R^1/R^o
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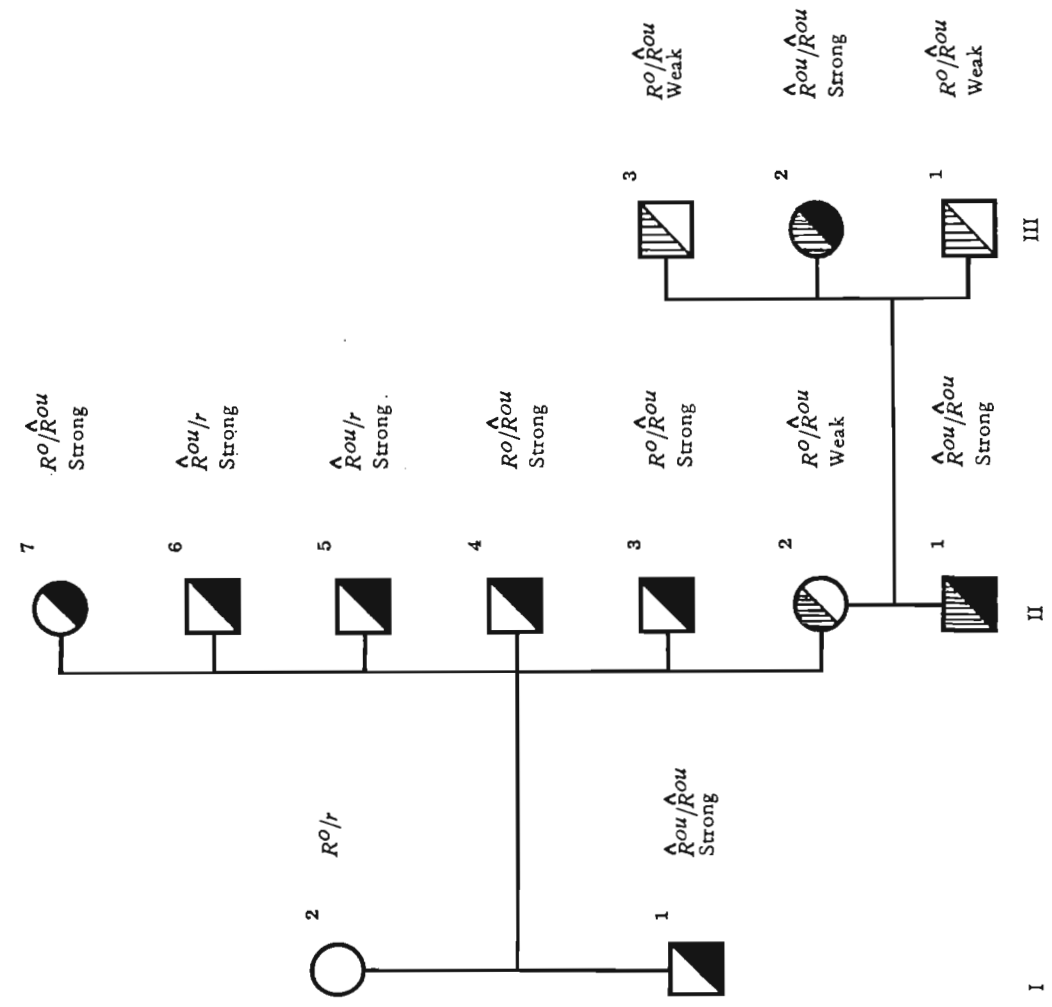
2
R^2/R^o
1
R^1/R^1
I
II
III


I
II
III
2
1
7
6
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4
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2
1
R^O/r
$\hat{R}^{ou}/\hat{R}^{ou}$ Strong
$R^O/\hat{R}^{ou}$ Strong
$\hat{R}^{ou}/r$ Strong
$\hat{R}^{ou}/r$ Strong
$R^O/\hat{R}^{ou}$ Strong
$R^O/\hat{R}^{ou}$ Strong
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$R^O/\hat{R}^{ou}$ Weak
$\hat{R}^{ou}/\hat{R}^{ou}$ Strong

Paper 55

Rh33 in two of three German siblings with D+ C+ c+ E− e+ red cells

P. MOORES, E. SMART, J. STERNBERGER, AND W. SCHNEIDER

The proposita in a German family of three siblings has D+ C+ c+ E− e+ f+ Rh:−17,19,33,34 red cells with weak C, e, f, Rh19, and Rh34 and stronger-than-usual Rh33 expression. One sibling has D+ C+ c+ E− e+ f+ Rh:17,19,33,34 red cells with weak f and ordinary-strength Rh33, and the other sibling has D+ C+ c+ E− e+ f+ Rh:17,19,−33,34 red cells. In the absence of any further family members, the proposita's unusual phenotype suggests that she has an R^{oHar} haplotype and a "new" Rh haplotype, provisionally named R^{tLisa}, that encodes Rh33, normal-strength D, weak C, weak or nondemonstrable e, Rh19, and Rh34, but not Rh17. Her Rh:33 sibling may have R^{1} and R^{oHar} and her Rh:−33 sibling R^{1} and r haplotypes. **TRANSFUSION** 1991;31:759–761.

THE R^{oHar} HAPLOTYPE was identified by Giles et al.[1] in 1971. Characteristically,[1-3] R^{oHar} produces difficult-to-detect D; no C, G, or E; normal c; weak e; some f; weak or no Rh17; no Rh18 or Rh19; and a low-frequency antigen numbered Rh33.

Other Rh haplotypes known[4-7] to encode Rh33 are $D^{IV}(C)-$ and R^{oJoh}. $D^{IV}(C)-$ produces enhanced but partial D, weak C, no e or f, and Go[a]; R^{oJoh} produces normal D and c, weak e, and no f.

Case Report

The proposita (I-1 in Fig. 1), from Hagen in western Germany, was born in 1912. She had never been pregnant or received a blood transfusion. Her sister (I-2), born in 1914, and her brother (I-3), born in 1920, were both healthy. In 1985, I-1 had an episode of unexplained hemolytic anemia for which no treatment was given or required. Her red cells gave normal positive results with anti-D and anti-c but unexpectedly weak positive or negative results with anti-C and negative results with anti-E and anti-e, which suggested a rare Rh phenotype. In 1989, when I-1 was healthy and her hematologic condition normal, her red cells gave essentially the same results. The family study is described.

Materials and Methods

Blood samples were received in anticoagulant from the proposita late in 1985, early in 1986, and at the end of 1989. Those from her two siblings were received early in 1986 only. The samples were all in good condition on arrival. A portion of each was stored in glycerol freezing solution at −32°C and another portion in liquid nitrogen. Tests with the remaining blood commenced within 24 hours. The routine grouping reagents were from the regular stocks of the Natal Blood Transfusion Service and from commercial sources in Germany and the United States. Two samples of anti-Rh33 Hawd. were available; they were earlier gifts from Dr. Carolyn Giles (Post Graduate School of Medicine, London, UK). Absorption was performed on one sample in Dr. Giles's laboratory until free of anti-A,B and anti-D; absorption of the other sample was similarly performed by the authors. Tests confirmed that both samples gave negative results with A_1, B, and D+ C− c+ E+ e− red cells. The anti-f was a local reagent that had given reliably good results for many years. Other rare reagents and red cells used were gifts from colleagues and Serum Cells and Rare Fluids members or were acquired locally. We employed standard techniques.[8] Wherever possible, we used single-dose controls or those known to give weak positive results. The enzymes employed were bromelin and ficin; the antiglobulin reagents were monospecific anti-IgG and anti-complement and polyspecific anti-IgG plus anti-complement.

Results

In 1989, the red cells of I-1 gave negative results in direct antiglobulin tests with anti-IgG and microscopic positive results with anti-complement reagents. We also obtained negative results with her red cells and *Arachis hypogea, Glycine soja, Salvia sclarea,* and *Salvia horminum* lectins and AB serum,

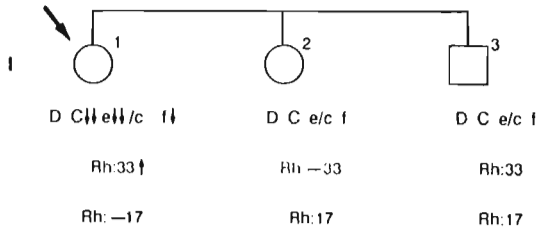

FIG. 1. The Rh groups of I-1 and her siblings. The up arrow indicates increased expression and the down arrows indicate decreased expression of the antigen to their left; two arrows indicate greater depression than one arrow. The red cells of I-1 were group A_1, NSs, Jk(a+b−), Sd(a−); those of I-2 were group A_1, MS, Jk(a+b+), Sd(a+), and those of I-3 were group B, MSNs, Jk(a+b+), Sd(a+). All three siblings had P_1+, Lu(a−), K−, Kp(a−), Le(a−b+), Fy(a+b+), Vel+, I+, Ge+, Wr(a−) red cells.

From the Natal Institute of Immunology and the Natal Blood Transfusion Service, Durban, South Africa, and the German Red Cross Blood Transfusion Service, Hagen Center, Hagen, Germany.

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Table 1. *Summary of results with family red cells and controls*

Reagents			Degrees of agglutination*			
			Red cells			
Number used	Anti-	Techniques	I-1	I-2	I-3	Positive control
1	-D	Sal†	(+)	1+	2+	2+
7	-D	Enz‡ & IAT§	3+	4+	4+	4+
1	-D inc‖	Sal	–	–	–	3+
1	-C	Sal	1+	NT¶	NT	3+
7	-C	Sal	–	NT	NT	3+
4	-C	Enz	2+	4+	4+	4+
1	-C	Enz	–	NT	NT	3+
8	$-C^w$	All	–	–	–	3+
9	$-rh_i$	All	–	3+	3+	3+
2	-G	Enz	2+	3+	4+	4+
6	-E	Sal & Enz	–	–	–	3+
2	-c	Enz	3+	3+	3+	3+
3	-e	Sal	–	3+	3+	3+
3	-e	Enz	1+	3+	3+	3+
1	-e	Enz	–	2+	(+)	3+
1	-f	Enz	1+	4+	3+	4+
3	-Rh19	Enz & IAT	–	NT	NT	3+
2	-Rh19	Enz	1+	3+	2+	3+
1	-Rh34	Enz & IAT	(+)	NT	NT	3+
3	-Rh34	Enz & IAT	–	3+	2+	3+
12	-Rh17	IAT	–	3+	3+	3+
1	-Rh29	IAT	3+	4+	NT	4+
1	-Rh33**	Sal	2+	–	1+	1+
1	-Rh33††	Enz	3+	–	1+	1+

* Agglutination ranges from strong positive to negative: 4+, 3+, 2+, 1+, (+), –.
† Saline test.
‡ Enzyme test.
§ Indirect antiglobulin test.
‖ Incomplete.
¶ Not tested.
** Hawd., absorption performed in Dr. Giles's laboratory.
†† Hawd., absorption performed in author's laboratory.

results that excluded polyagglutination and other forms of bacterial contamination. No blood group antibodies were detected in the proposita's serum. Table 1 shows the results of the Rh typing tests and Fig. 1 the Rh phenotypes and antigens identified in the family. The red cells of I-1 were D+ C+ c+ E– e+ f+ Rh:–17,19,29,33,34; those of I-2 were D+ C+ c+ E– e+ f+ Rh:17,19,29,–33,34; and those of I-3 were D+ C+ c+ E– e+ f+ Rh:17,19,29,33,34.

In tests and titrations, anti-D and anti-G reagents agglutinated the red cells of I-1 marginally less strongly than those of her siblings and the positive controls. The anti-C, -e, -Rh19, and -Rh34 reagents agglutinated her red cells weakly (see anti-C titration in Table 2) or not at all, and the anti-f reagent gave less than a single-dose score with them but gave single-dose scores with those of both siblings. An absorption-elution study with anti-C confirmed that the red cells of I-1 had C antigen. The titers and scores with anti-c and all three siblings' red cells were similar to those with the D+ C+ c+ E– e+ (i.e., single-dose c) control cells. The anti-Rh33 reagents gave strikingly stronger positive results with the red cells of I-1 than with those of I-3 and three known positive control samples.

The red cells of all three siblings gave negative results with anti-D^w, -Rh30 (-Go^a), -Rh32, and -Rh37 (Evans). Positive control cells unfortunately were not available.

Discussion

The enhanced Rh33 and weaker C, e, f, Rh19, and Rh34 antigens of I-1 in relation to those of I-3, whose genotype we believe to be R^1/R^{oHar}, and her Rh:–17 phenotype at first suggested that I-1 is homozygous for R^{oHar}. The f+ result with I-3 and our anti-f reagent was not surprising, for R^{oHar} was known to produce this antigen. Moreover, in family H.H. from Giles et al.,[1] the red cells of II-3, whose genotype was also R^1/R^{oHar}, were seen to be weak with four sera (anti-f) by enzyme techniques but had near-normal strength with two sera by antiglobulin technique. On the other hand, I-1 might have R^{oHar} and an unusual *DCe* haplotype that encoded Rh33, or she might have an unusual modifying gene that suppressed C, e, f, Rh17, Rh19, and Rh34 but had little or no effect on D, G, and c and that elevated Rh33.

Homozygosity for R^{oHar} in I-1 was unlikely, as she had C+ red cells. An unusual modifying gene was also unlikely, as one that suppressed some and yet enhanced other Rh antigens was difficult to imagine. The proposita might, however, have R^{oHar} and a "new" haplotype, named by us "R^{1LLsa}." If that is the true explanation of the phenotype of I-1, the products of R^{1LLsa} can be seen to be normal or near-normal D and G, weak C and e, probably no f, no Rh17, and normal Rh33. A $D^{IV}(C)-$ haplotype was excluded by her c, f, and nonenhanced D antigens and Rh:–17 phenotype, and an R^{oJoh} haplotype by her C antigen and Rh:–17 phenotype.

An R^{LLsa}/r, instead of an R^1/R^{oHar} genotype, in I-3 was unlikely. Although the presence of *r* would explain his f antigen, his red cells gave normal positive results with anti-C and $-rh_i$.

In view of the findings, the four haplotypes presumably present in the deceased parents of these three siblings were R^1, *r*, R^{oHar}, and R^{1LLsa}.

Table 2. *Anti-C titration by one-stage enzyme technique*

Red cells	Anti-C dilutions							
	1	2	4	8	16	32	64	128
I-1	2+	1+	–	–	–	–	–	–
D+ C+ c+ E+ e+ control	3+	3+	3+	3+	1+	1+	(+)	–
D+ C– c+ E– e+ Rh33 control	–							
D– C– c+ E– e+ control	–							

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Phyllis P. Moores, PhD, Head, Immuno-Haematology Research Laboratory, Natal Institute of Immunology, P.O. Box 2356, 4000 Durban, South Africa. [Reprint requests]

Elizabeth Smart, MT, Section Supervisor, Serology, Natal Blood Transfusion Service, Durban, South Africa.

Joachim Sternberger, MD, Head, Medical Department, German Red Cross Blood Transfusion Service.

Waldemar Schneider, MD, Professor and Medical Director, Medical Department, German Red Cross Blood Transfusion Service, Hagen Center, Hagen, Germany.

Paper 52

The Rh haplotype *D*– – identified in five Cape Colored families

To the Editor:

The *D*– – gene encodes enhanced D but no serologically detectable C c E or e antigens on red cells.[1] *D*– – homozygotes have been reported, rarely, in whites, blacks, Hindu Indians, Puerto Ricans, and Japanese. We detected the heterozygous *D*– – state in the propositi and 16 of 31 members in four Cape Non-Malay and one Cape Malay Colored families in Cape Town, South Africa, after noticing apparent maternal or paternal exclusions in parentage disputes. The Cape Non-Malay Coloreds have 22 percent Asian, 46 percent black and Khoisan, and 32 percent white genes, and the Cape Malay Coloreds have 42 percent Asian, 25 percent black and Khoisan, and 33 percent white genes.[2] The Khoisan are aboriginal African pastoralists and hunter-gatherers who were formerly known, respectively, as Hottentots and Bushmen.[3]

The rare Rh haplotype $dCce^{s}R^{-34}$ ($\dot{r}'^{s}$) was identified in trans to *D*– – in one and the rare Rh haplotype $DceR^{-18}$ in trans to *D*– – in another of the Cape Non-Malay Colored families (Figs. 1 and 2). The rare Rh haplotype $DceR^{-34}$ ($\dot{R}^{o}$) was identified in trans to *D*– – in the Cape Malay Colored family. The other haplotypes found in trans to *D*– – were *Dce* or *dce*, *DCe*, and *DcE*. The *D*– – heterozygotes' red cells gave 1 + to 3 + reactions with incomplete anti-D known to detect enhanced D antigen in saline tests. One anti-D was from Gamma Biologicals (Houston, TX), and the other five had been selected and standardized in Durban using Dantu + and red cells from En^{a}/En heterozygotes as positive and DcE/DcE red cells as negative controls. The *D*– – heterozygotes' red cells also gave lower titers and scores than those with similar phenotypes in persons with ordinary *Rh* genes in titrations with antibodies to C c E e Rh18 and Rh34 (as relevant). All findings were confirmed using repeat blood samples. Negative results were obtained with the *D*– – heterozygotes' red cells and five anti-Rh32 (one supplied by Gamma Biologicals; positive control red cells were unfortunately not available), one anti-Rh40 (anti-Tar), one anti-Rh30 (anti-Go^{a}), and two anti-M^{g}. The corre-

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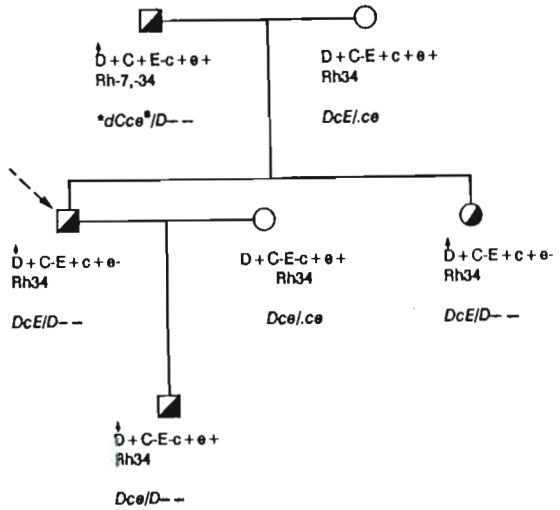

FIG. 1. Cape non-Malay family in which one person has *D--/dCce^{s}R^{-34}* ($\bar{\bar{R}}^{o}/\dot{r}^{s}$) genes. The half-shaded characters represent the presence of the *D--* gene. The arrow over the D indicates enhanced reactivity of that antigen. The asterisk before the *dCce^{s}* gene indicates that the gene does not encode Rh34.

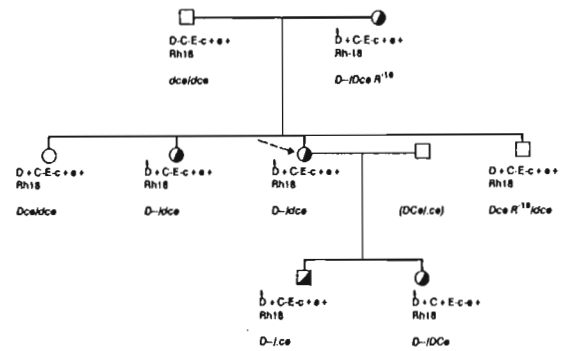

FIG. 2. Cape non-Malay family in which one person has *D--/DceR^{-18}* ($\bar{\bar{R}}^{o}/\hat{R}^{o}$) genes. The half-shaded characters represent the presence of the *D--* gene. The arrow over the D indicates enhanced reactivity of that antigen.

sponding antigens, the presence of which is associated with positive results with incomplete anti-D in saline tests, were therefore excluded. Negative results with *Arachis hypogea* and *Glycine soja* lectins and AB serum excluded the *D--* heterozygotes' red cells from being polyagglutinable.

No previous accounts of D+ C+ c+ e^{s}+ Rh:−34 ($\dot{r}^{s}R_o$) or D+ c+ e+ Rh:−34 ($\dot{R}_o$) red cells due to the genotypes *D--/r'^{s}* or *D--/R̂°*, respectively, or of D+ c+ e+ Rh:−18 red cells due to the *D--/R̂°* genotype, exist in the literature. Because our findings have shown that *D--* exists at the Cape, paternity testing laboratories in South Africa are urged to include it confidently among rare *Rh* alleles encountered in their populations.

MAUREEN MULVIHAL, MT
Western Province Blood Transfusion Service
Cape Town, South Africa
PHYLLIS MOORES, PHD
Natal Institute of Immunology
Durban, South Africa

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Paper 54

Hum Hered *862*

D-- and *Dc*- Gene Complexes in the Coloureds and Blacks of Natal and the Eastern Cape and Blood Group Phenotype and Gene Frequency Studies in the Natal Coloured Population

Phyllis Moores[a], *Ulla Vaaja*[b], *Elizabeth Smart*[a]

[a] Natal Blood Transfusion Service and Institute of Immunology, Durban, and [b] Eastern Province Blood Transfusion Service, Port Elizabeth, South Africa

Key Words. *D*-- gene complex · *Dc*- gene complex · 'Deleted' Rh gene complexes · Blood group phenotype frequencies · Blood group gene frequencies

Abstract. Following the detection of apparent exclusions in the Rh system in two Coloured (mixed race) families during paternity testing, a rare *D*-- gene complex was identified in one family and two examples of an unusual gene complex producing weak e and very weak or non-demonstrable f antigen in the other. The latter, which almost certainly belong to the heterogeneous collection known as *Dc*-, were found when the Rh phenotypes expected to give f+, instead gave f- or f+^{W} (weak positive) results and those expected to give f-, gave f+^{W} results. Blood group phenotype and gene frequency studies showed that the Natal Coloured population contains a mixture of approximately 40% Black, 30% White and 30% Indian (Asian) genes. The phenotypes A_1 high H, B high H, B low H, K+ and Kp(a+) associated with Caucasoids and the phenotypes A_{bantu}, Dantu+, hr^{S}- (Rh: -18,-19), hr^{B}- (Rh: -31,-34) and Fy(a-b-) associated with Negroids were all represented. The *DCe/Dc*- frequency was 6.9% and the *DcE/Dc*- frequency 2.6%.

Introduction

The first example of the rare Rh gene complex *D*-- was recorded by Race et al. [1] in 1951. The propositus, who was believed to have homozygous *D*--/*D*-- genes, had enhanced D but no serologically detectable C, c, E or e antigens on his red cells. The first South African examples were recorded by Mulvihal and Moores in 1991 [2] in 5 Coloured (mixed race) families living near Cape Town.

The rare gene complex *Dc*- was first described by Tate et al. in 1960 [3] in an inbred family of French extraction. The red cells of the propositus and 2 of his siblings had enhanced D, normal strength but qualitatively different c and no serologically detectable E or e antigens. A similar complex was recorded in an American

Black family by Leyshon [4] in 1967, and one in another family by Yamaguchi et al. [5] in 1969. In 1980, after studying the serological results with the red cells of two further American Blacks, Tessel et al. [6] suggested that all the *Dc–* complexes found so far could be included in a single heterogeneous group. Some made weak e and normal f, others made weak e and weak f and still others made no e or f antigens.

This paper describes the results obtained with 2 Coloured families in Natal and the Eastern Cape which showed that one family had a *D––* and the other had two *Dc–* gene complexes. In the Natal Coloured population, blood group phenotype and gene frequency studies also disclosed that the *Dc–* gene was not rare.

Materials and Methods

The blood samples were of clotted and unclotted blood and not more than 3 days old when first tested. In the frequency studies, as far as possible, random distribution was assured by including only samples from males and unmarried females who were making their first donations. A portion of each sample was stored in glycerol freezing solution at –30 °C and recovered by dialysis against saline when required. Prior to testing, the red cells were washed 3 times with freshly made saline, and 2–5% suspensions were prepared in the preserving fluid of Burgess and Vos [7] for use.

In the family studies, the anti-D, -C, -E, -c and -e reagents used were from both commercial and local sources. The anti-C^w, -rh_i, -G, first of 2 anti-f, 6 anti-hr^S and 3 anti-Rh34 were from local donors. The second of two anti-f and the anti-Be^a, -C^x, -D^w, -Go^a, -V, -VS, -Rh17, -Rh32, -Rh33, -Rh40 and -M^g were much valued gifts from SCARF and other colleagues. The techniques recommended by their suppliers were adhered to strictly. Where possible, more than one example of each reagent was included; their specificity and potency were verified with known positive and negative control red cells with every batch of tests. The results with the local reagents by tube technique were read both macro- and microscopically after light centrifugation. To detect enhanced D antigen, suitable local incomplete anti-D were standardised for use by saline technique. The positive controls for this were DCe/DcE Dantu+ and DCe/DcE (En(a+) and the negative controls DcE/DcE and dce/dce red cells.

In the frequency studies, the ABO groups of the Coloured donors were determined with anti-A, -B, -A,B and -A_1. The H status of the A, B and AB donors was identified in saline titrations using *Ulex europaeus* anti-H lectin and that of the O donors with 'Bombay' anti-A+B+H. The reverse grouping was made with A_1, A_2, B and O red cells. The donors' MNSs phenotypes were determined with both rabbit and human anti-M and anti-N, *Vicia graminea* anti-N lectin and with anti-S, -s and -U. To identify heterozygous where homozygous *MM* and *NN* genes were expected, selected human anti-M and anti-N were diluted to agglutinate phenotype MM and NN red cells only. Other reagents employed were anti-Henshaw, anti-Ny^a and a serum known to contain anti-S, -Dantu, -Mur, -Hut, -Bp^a, -Di^a, -Milne, -Pt^a, -Rb^a, -Sw^a, -Tr^a, -Wd^a and -Wr^a. The Rh groups of the donors were determined with anti-D, -C, -C^w, -rh_i, -E, -c, -e, -hr^S, -Rh34 and-f. Increased D antigen was detected with incomplete anti-D used by saline technique, as already described. The Kell groups of the donors were determined with anti-K, -k, -Kp^a and -Kp^b and their Duffy groups as far as possible with anti-Fy^a and -Fy3. *Arachis hypogea* and *Glycine soja* lectins and AB serum were included to ensure that no results were false due to bacterial contamination. The urea lysis test of Heaton and McLoughlin [8] was employed to detect Jk(a–b–) red cells.

Standard blood grouping techniques were used [9]. The enzyme used was bromelin. A sufficient quantity of the freeze-dried material was reconstituted as required and stored at 4 °C until completed or expired.

Bernstein's formula, quoted by Glass and Li [10], was employed to assess the mixture of Black, White and Indian blood group genes in the Coloured donors. The percent genes contributed by the Natal Blacks was calculated from the *Dce* gene complex frequency in the Coloureds. The percent genes contributed by the Natal Whites and Indians were calculated using the blood group gene frequencies recorded earlier in these populations by Moores [11, 12].

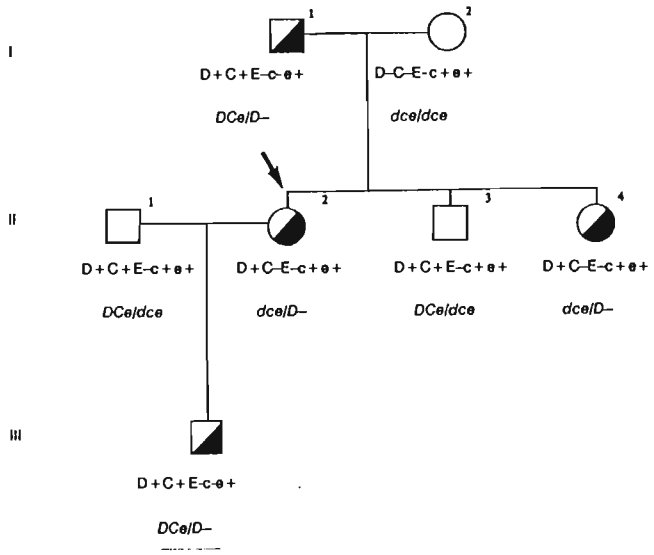

Fig. 1. Pedigree of the Coloured family with a *D*– – gene. The half black squares and circles indicate the heterozygotes and the arrow points to the proposita.

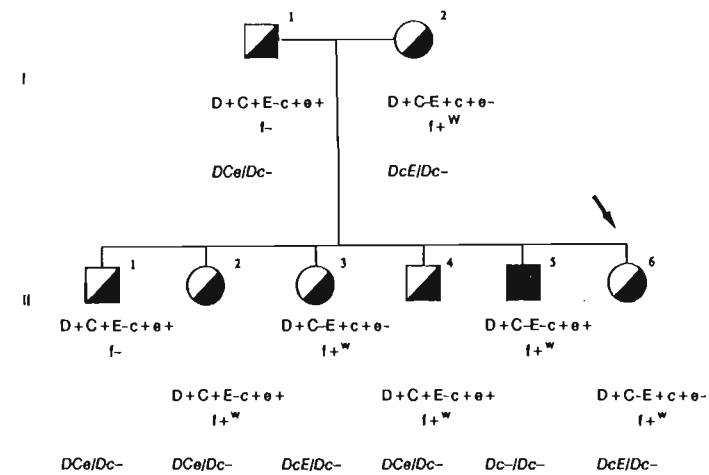

Fig. 2. Pedigree of the Coloured family with two *Dc*– genes. The black square indicates the apparent homozygote and the half black squares and circles the heterozygotes. The arrow points to the proposita.

Results

Family Studies

The 2 Coloured families described here were found during disputed paternity testing when difficulties were experienced in identifying the Rh genes from the Rh phenotyping results. In the first family (fig. 1), the D+C–E–c+e+ red cells of the proposita, II-2, appeared to exclude her as the mother of III-1 who had D+C+E–c–e+ red cells. Moreover, I-1, who had D+C+E–c–e+ red cells, also appeared excluded as the father of II-2 and II-4 with

Table 1. Results with Rh antisera and the red cells of the family members in figure 2

Family member	Red cell phenotypes	Grouping reagents									
		IgM D	-C	-E	-c	-e	-f	1-s -hr^s	2-s -Rh34	-hr^i	G
I-1	D+C+E-c+c+	2	4	–	3	2	–	(3)	3	2	3
I-2	D+C-E+c+c-	3	–	2	4	(2)	1	–	(3)		3
II-1	D+C+E-c+e+	3	+	–	3	2	–	(2)	3	2	NT
II-2	D+C+E-c+e+	3	+	–	3	2	–	1	3	2	NT
II-3	D+C-E+c+e-	4	–	2	3	(1)	1	–	(1)		NT
II-4	D+C+E-c+e+	3	4	–	3	2	–	(3)	3	1	NT
II-5	D+C-E-c+e+^{w}	3	–	–	3	(3)	1	–	(1)		NT
II-6	D+C-E+c+c-	3	–	2	4	(2)	(3)	–	(1)		3
Controls											
D+C+E-c+c+		3	4		3			1	4	2	3
D+C+E-c-e+					–						
D-C+E-c+e+f+							1				
D+C-E+c+c-f-				3	4	–	–				
D+C-E+c+e+				2		4					
D-C-E-c+e+		–	–	–		4	4	4	1		–
D+C-E-c+c+ Rh:-18								–			
D-C+E-c+e+ Rh:-34									–	–	

1-s = One-stage; 2-s = two-stage enzyme technique. Reading code (from strong to weak): 4, 3, 2, 1, (3), (2), (1), +.

D+C-E-c+e+ red cells. Enhanced D antigen was detected on their cells. The findings were explained by postulating that the affected family members had a *D--* in trans to their ordinary Rh gene complexes. Negative results with a serum containing anti-Rh32 excluded an R^N complex. Negative results with anti-C^w, -D^w, -C^x, -Be^a, -Go^a, -Rh33, -Rh40 and -M^g also excluded all the corresponding genes. Anti-Rh37 (Evans), specific for D••, was unfortunately not available. Figure 1 shows that II-2 and II-4 had *D--/dce* gene complexes.

In the second family, figure 2 and table 1 show that I-2, II-3 and II-6 had D+C-E+c+c- red cells. Instead of negative results with anti-f, however, their cells gave f+^{w} (weak positive) results. Furthermore, the red cells of I-1, II-1, II-2 and II-4 (D+C+E-c+e+) and those of II-5 (D+C-E-c+e+^{w}), instead of normal positive results with anti-f, gave negative or f+^{w} results. These findings, which were confirmed with repeat blood samples, were explained by postulating that both I-1 and I-2 had a *Dc-* in trans to their ordinary Rh gene complexes. In each of their children, the complex produced either a small amount of or non-demonstrable f antigen. The amount of f was not necessarily the same as in the parent from whom the complex had been inherited and bore no rela-

tionship to the Rh type of the complex that the child had in trans. Due to the small supply of anti-f, no attempt was made to confirm the f– results in absorption-elution studies.

In titrations with anti-E, the red cells of I-2, II-3 and II-6 (D+C–E+C+e–) gave weaker positive results than the known DcE/DcE control This suggested that the members had *DcE/Dc–* genes.

In titrations with anti-c, the red cells of I-1, II-1, II-2 and II-4 (D+C+E–c+e+) gave similar results to the known DCe/dce control. The red cells of I-2, II-3, II-6 (D+C–E+c+e–), and those of II-5 (D+C–E–c+e+^{w}), also reacted to the same titre as the known D+C–E+c+e– control. The two *Dc–* complexes in this family therefore encoded normal c antigen.

The red cells of I-2, II-3 and II-6 (D+C–E+c+e–) reacted weakly with 4 of 10 anti-e reagents. These findings were confirmed later in titrations. Marginally stronger results were obtained with the red cells of II-5 (D+C–E–c+e+^{w}). In addition, an IgM anti-e giving titres of 32 (score 29–38) with the red cells of I-1, II-1, II-2 and II-4 (D+C+E–c+e+) and a titre of 64 (score 54) with the heterozygous E/e control, gave negative results with his cells. The presence of e antigen in II-6 was confirmed by absorption-elution study (insufficient cells for similar studies were available from I-2 and II-3). The red cells of I-2, II-3 and II-6 (D+C–E+c+e–) and those of II-5 (D+C–E–c+e+^{w}) reacted weakly with anti-Rh34 and gave negative results with anti-hr^{s}. These findings accorded with the weakness of their e. The antigens produced by the *Dc–* complexes in this family were therefore weak e, weak Rh34 and non-demonstrable hr^{s}.

The D antigen of all the family members tested, including that of II-5, who evidently had *Dc–/Dc–* genes, gave weak positive results with incomplete anti-D reagents used by saline technique. This confirmed that their *Dc–* complexes encoded enhanced D antigen.

Negative results were obtained with the red cells of I-1 and I-2 and anti-C^{w}, -Go^{a}, -V, -VS, -Rh33, -Tar, -D^{w}, -Rh32 and anti-G. The red cells of II-3 and II-5 gave positive results with anti-Rh17 and those of I-1, II-2 and II-4 positive results with anti-rh$_{i}$. The family denied knowledge of consanguinity.

Dc– complexes were also identified in a third Coloured family, 4 Black families and 1 Black individual. Three propositi had D+C–E+c+e– red cells which reacted weakly with anti-f. Their children had D+C–E–c+e+ red cells, but the normal *Dce* gene that each child had inherited in trans made their f results uninformative. Two propositi had D+C–E–c+e+ red cells which reacted strongly with anti-f. The D+C–E+c+e– red cells of their children reacted with this antibody weakly. The Black individual had D+C–E–c+e+^{w}f–hr^{s}– LW(a+) Rh:17,w34 red cells. As no absorption-elution studies were done and a family study was not possible, like II-5 in family 2 he was presumed to have homozygous *Dc–* gene complexes.

The frequencies of the phenotypes, D+C+E–c+e+f– and D+C–E+c+e–f+^{w}, were estimated in 229 random D+ Black blood donors. No f– were found among 32 donors with D+C+E–c+c+ red cells and only one f+^{w} among three donors with D+C–E+c+e– red cells. Serological and antigen dosage studies suggested that this donor had *DcE/Dc–* genes.

Table 2. ABO phenotype and gene frequencies in the Natal Coloured population

Group	n	%	Gene	Gene frequencies	Variants		
					type	n	phenotype, %
A_1	149	27.54	p1	0.154	$A_1H\uparrow$	14	2.59
A_2	38	7.02	p2	0.054	$BH\uparrow$	5	0.92
A_{bantu}	15	2.77	pbantu	0.015	$BH\downarrow$	2	0.37
B	90	16.66	q	0.211			
A_1B	19	3.51	r	0.567	Total	21	
A_2B	12	2.21					
A_{bantu} B	5	0.92	Total	1.001			
0	213	39.37					
			$\chi^2 = 0.09$ (for 1 d.f.)				
			$p = 0.8$				
Total	541	100.00					

Table 3. MNSs phenotype and gene frequencies in the Natal Coloured population

Group	n	%	Gene	Gene frequencies	Henshaw+		Variants	
					Group	n	type	n
MS	11	2.08	MS	0.~~1752~~ 1465	Ms	1	Dantu+	3
MSs	58	10.98	NS	0.~~0833~~ 1120	MSs	6	Mi. II	1
Ms	86	16.29	Ms	0.~~3741~~ 4027	MNS	6	Mi. III	4
MNS	32	6.06	Ns	0.~~3674~~ 3388	MNs	1		
MNSs	91	17.24			NSs	1		
MNs	147	27.84	Total	1.0000				
NS	5	0.95			Total	15		
NSs	28	5.30	$\chi^2 = \frac{0.057}{0.001}$ for M and N					
Ns	70	13.26	$p = 0.98$ $<0,5$ (1.df)					
			$\chi^2 = 19,18$ for MNSs					
Total	528	100.0	$p < 0,01$ (5 d.f.)					

Phenotype and Gene Frequency Studies

The ABO, MNSs, Rh, Kell and Duffy phenotype and gene frequencies were estimated in approximately 500 Natal Coloured blood donors. The results are shown in tables 2–6. In the Rh system the D^u were included with the D+ samples. The predominantly Caucasoid phenotypes A_1 high H, B high H and B low H, Mi.II and Mi.III, K+ and Kp(a+), and the predominantly Negroid phenotypes A_{bantu}, Henshaw+ and Dantu+, hr^S– (Rh:–18,–19) and hr^B– (Rh:–31,–34), and Fy(a–b–), were all identified. No S–s–U–, Jk(a–b–) or Ny(a+)

Table 4. Rh phenotype frequencies in the Natal Coloured population

Phenotype	Probable genotype		n	%
D+C+E−c−e+	*DCe/DCe*		64	
D+C+C^{w}+E−c+e+	*DC^{w}e/DCe*		1	
D^{u}C+E−c−e+	*D^{u}Ce/dCe*		4	
		Subtotal	69	12.75
D+C+E−c+e+f+	*DCe/dce*		170	
D+C+E−c+e+f−	*DCe/Dc−*		14	
D+C+E−c+e+f+	*dCce^{s}/Dce*		4	
D+C+C^{w}+E−c+e+	*DC^{w}e/dce*		11	
D^{u}C+E−c+e+	*D^{u}Ce/dce*		2	
D^{u}C+E−c+e+	*dCce^{s}/D^{u}ce*		1	
D−C+E−c+e+ Rh: −34	*dCe^{s}* R^{-34}/Dce R^{-34}		1	
		Subtotal	203	37.52
D+C−E+c+e+f+	*DcE/dce*		58	
D+C−E+c+e+f−	*DcE/Dc−*		2	
D+C−E+c+e+ Rh: w34	*DcE R^{w34}/Dce R^{-34}*		4	
D^{u}C−E+c+e+	*D^{u}cE/dce*		2	
D+C−E+c+e+ Rh: −19	*DcE/Dce R^{-19}*		2	
		Subtotal	68	12.57
D+C−E+c+c−	*DcE/DcE*	Subtotal	8	1.48
D+C−E−c+e+	*Dce/Dce*		106	
D+C−E−c+e+ Rh: −34	*Dce R^{-34}/Dce R^{-34}*		5	
D+C−E−c+e+	*Dce/Dc−*		2	
D^{u}C−E−c+e+	*D^{u}ce/dce*		6	
		Subtotal	119	22.00
D+C+E+c+e+	*DCe/DcE*		34	
D+C+E+c+e+ Rh: w34	*dCce^{s} R^{-34}/DcE R^{w34}*		1	
		Subtotal	35	6.47
D−C+E−c+e+	*dCe/dce*		3	
D−C+E−c+e+	*dCce^{s}/dce*		5	
		Subtotal	8	1.48
D+C+E+c−e+	*DCe/DCE*	Subtotal	3	0.55
D−C−E−c+e+	*dce/dce*	Subtotal	28	5.18
		Total	541	100.00

Table 5. Rh gene frequencies and *Dc*– heterozygotes in the Natal Coloured population

Rh system gene frequencies		*Dc*– heterozygotes			
		phenotype	n	total	%
DCe	0.3258	D+C+E–c+e+	14	203	6.9
dCe	0.0325				
DCE	0.0077	D+C–E+c+e–	2	76	2.6
DcE	0.1067				
dcE	0.0000				
Dce	0.2971				
dce	0.2302				
Total	1.0000				

$\chi^2 = 0.47$ (for 5 d.f.); $p = 0.99$.

Table 6. Phenotype and gene frequencies in the Kell system and Duffy phenotypes in the Natal Coloured population

Group	n	%	Gene frequencies	
Kell system				
K+k+	21	3.88	K	0.0194
K–	520	96.12	k	0.9806
Total	541	100.00		1.0000
Kp(a+b+)	2	0.37	Kp^a	0.0018
Kp(a–)	537	99.63	Kp^b	0.9982
Total	539	100.00		1.0000
Duffy system				
Fy(a+)	295	54.73		
Fy(a–)	65	12.06		
Fy(a–b+)	134	24.86		
Fy(a–b–)	45	8.35		
Total	539	100.00		

red cells were detected. The frequency of *Dc*– gene complexes in the Coloureds with D+C+E–c+e+ red cells was 6.9% and in those with D+C–E+c+e– red cells 2.6%.

As the known *Dce* gene complex frequency in the Natal Blacks was 64.3% and in the Natal Whites and Indians negligible [11, 12], calculations using the frequency found in the Natal Coloureds in this study showed that approximately 40% of the blood group genes in the Coloureds were of Natal Black origin. Calculations using the *A*, *B*, *0*, *MS*, *Ms*, *Ns*, *DCe* and *dce* frequencies in the Natal Whites and Indians also showed that 30% of the remainder was of White and 30% of Indian origin. The Natal Coloureds therefore had a mixture of approximately 2 parts Natal Black, 1.5 parts Natal White and 1.5 parts Natal Indian genes.

Discussion

Coloured people first appeared in Natal in the early part of the 19th century some time after Whites from Western Europe who had travelled north from the Cape of Good Hope had succeeded in establishing a trading centre there [13, 14]. The ancestors of the Zulu Black people are believed to have journeyed south from

East Africa. It is thought that they arrived in Natal more or less at the time that Whites first set foot ashore at the Cape. Large numbers of Indians were imported from India into Natal from the year 1860 to work on the sugar plantations. Many elected not to return to India when their period of indenture was over. The majority of the earliest Indians to arrive were males, but when Indian women came, their social customs were restored [13]. For many years the Zulus resented the presence of the Indians. As a result, the mixture of blood group genes in the Coloured population was expected to represent predominantly Black-White and Indian-White rather than Black-Indian miscegenation.

In this study, the Natal Coloureds were found to have higher q (*B*), *DCe* and *dce* and lower *Dce* frequencies than the Cape Coloureds and Coloureds living in the Transvaal [14–16]. The χ^2 for Rh gene complex frequencies in the Natal versus the Cape Coloureds was 29.47 for 6 degrees of freedom. This confirmed that a real difference exists between them. The q (*B*), Henshaw and Fy(a–b–) frequencies in the Natal Coloureds were lower than in the Natal Blacks [11] and their *MS*, *DCe*, *dce* and *K* frequencies lower than in the Natal Whites and Indians [12]. As in the Natal Blacks, Henshaw was associated primarily but not exclusively with *MS*. The approximately 40% Black, 30% White and 30% Indian blood group genes in the Natal Coloureds showed that all three populations had made substantial contributions to the Coloured gene pool.

The *D*–– gene complex is well known [17]. Its discovery in the Natal and Eastern Cape Coloured families complements those found in the Coloured families at the Cape. The origin of this gene in South Africa remains uncertain.

The *Dc*– gene complex has not been recorded before from South Africa. It was identified when Rh phenotypes which normally give positive results with anti-f unexpectedly gave negative or weak positive results; also when red cells which normally give negative results with anti-f unexpectedly gave weak positive results. The evidently homozygous *Dc*–/*Dc*– family member's red cells (II-5 in fig. 2) had minimally stronger f antigen than those of his heterozygous parents and siblings. The antigens encoded by the *Dc*– complex were enhanced D, normal strength c, weak e, weak or non-demonstrable f, weak Rh34 and non-demonstrable hr^S.

Tessel et al. [6] noted that the known *Dc*– gene complexes produced variable amounts of e and f antigens. The amount of e produced by the Natal and the Eastern Cape *Dc*– complexes was weak. The amount of f produced varied in different family members irrespective of both the quantity expressed in the parent from whom the gene had been inherited and any possible influence by the Rh type of the complex inherited in trans. The *Dc(e)* complex in a Black woman reported by Issitt et al. [18] differed in that enhanced D was not encoded and the complex in trans expressed reduced e antigen.

As so few *Dc*– complexes have been recorded elsewhere [17], yet they were identified in this study in an appreciable number of Natal Coloured donors, in Natal at least the complex must be associated primarily with the Black population. Only the scarcity of anti-f may have prevented other examples from having been recorded

from South Africa before. The higher *Dc–* frequency in donors with D+C+E–c+c+ than D+C–E+c+e– red cells may have been due to the complex being recognised more easily in the former compared with the latter. In South Africa, *Dc–* clearly has implications in disputed paternity and other family blood group inheritance studies.

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Phyllis Moores, PhD
Head, Immuno-Haematology Research Unit
Natal Institute of Immunology
P.O. Box 2356
4000 Durban (South Africa)

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Rh_{null} Red Cells and Pregnancy

B. J. Gibbs, Phyllis Moores

Western Province Blood Transfusion Service, Cape Town, and Natal Blood Transfusion Service, Durban, South Africa

Abstract. The haemoglobin, haematocrit and osmotic fragility red cell values in a South African white woman with Rh_{null} cells and the corresponding haematological syndrome were shown to vary only minimally during her third pregnancy. This occurred in spite of the precautionary donation by her of two units of her blood at 20 and at 27 weeks of pregnancy for storage in liquid nitrogen. Although there was fear to the contrary, the woman's infant was found at birth to be suffering only mildly from haemolytic disease of the newborn due to the anti-Rh29 antibodies present in her plasma.

Introduction

Rh_{null} red cells lack all serological evidence of Rh gene expression. This condition arises due to the inheritance of either two very rare amorphic $\bar{r}$ (or ---) genes at the Rh (CDE) chromosomal locus or two rare suppressor or 'regulator' $X^{o}r$ genes which prevent expression of normal Rh genes. The expression of the *SsU* genes and the genes responsible for the Ii antigens is modified as well and the individuals usually exhibit a mild to moderate form of anaemia known as the Rh_{null} syndrome [1]. When one $X^{o}r$ gene is inherited, the Rh antigens on the red cells may be partially suppressed.

Although many workers have described in detail the red cells and anaemia in Rh_{null} persons, little has been published about the effect of pregnancy on the haemoglobin level of an Rh_{null} woman or concerning the effect of anti-Rh29 antibodies in an infant. In 1980, with the discovery of the first Rh_{null} woman in South Africa, these studies become possible and our findings are recorded in this paper.

Case Report

Susannah F. was a white women of 30 years whose family had lived near Cape Town for a number of generations. She was found through a routine antenatal test when her red cells were seen not to be agglutinated (confirmed in absorption-elution tests) by a large number of different Rh reagents, including three with anti-Rh17, one with anti-Rh29 and six with anti-LW speci-

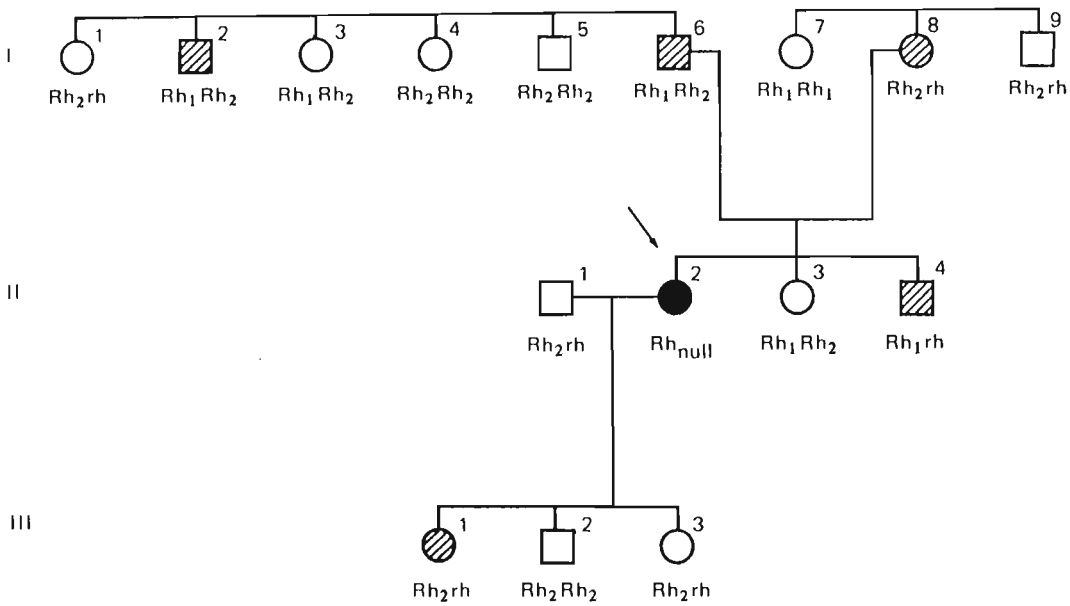

Fig. 1. Weak Rh antigens (⊘, ▨) in Susannah F. family members. ↘ = Proposita.

ficity. In addition, her cells were typed as O, Ms, U–, P_1, Lu(a–b+), K–k+, Kp(a–b+), Js(a–b+), Le(a–b+), Fy(a–b+), Jk(a+ b–), I+, i weak+, Wr(a–b+) Ge+, Vel+, Sc:–1,2, Sk(a–), Sd(a–) confirmed 4 months after delivery, Hil–, Henshaw–, M_1–, Do(b+) and Xg(a+). Red cell antigen dosage titration studies revealed reduced s antigen expression when compared with homozygous (ss) control cells. Anti-Rh29 antibodies were identified when her serum was found to agglutinate by enzyme and indirect antiglobulin techniques all cell samples tested including those negative for a large number of high frequency antigens. Only Rh_{null} cells remained unagglutinated.

The family Rh groups showed no evidence of the presence of $\bar{f}$ (---) genes. However, red cell antigen dosage titration studies with various Rh reagents revealed that the cells of Susannah's uncle (I–2), father (I–6), mother (I–8), brother (II–4) and daughter (III–1) had some Rh antigens expressed weakly. This was taken as evidence that Susannah had 'regulator' type Rh_{null} cells (fig. 1).

Obstetrically, Susannah was gravida III, para 2, and she had no history of past pregnancy complications or blood transfusions. She gained weight normally throughout her third pregnancy, her blood pressure was normal and she remained clinically well. Karyotyping showed that she had the normal 46XX complement of chromosomes. At 20 weeks of pregnancy, a sonar examination of her uterus revealed the presence of a single fetus with the placenta situated in the left lateral position.

Results

The Proposita

No significant changes were noted in haemoglobin and haematocrit values obtained at intervals during Susannah's pregnancy and after delivery. The initial mean cell haemoglobin concentration was 0.35% and reticulocyte count 30 per 1,000 RBCs. Mild stomatocytosis only was noted in the blood films. The osmotic fragility test (fig. 2) with her cells remained consistently mildy increased throughout pregnancy, and at 4 months post partum. In view of the report by

Bar Shany et al. [2] of 2 Rh_{null} women elsewhere who had normocytic, normochromic anaemia and Hb values of 8 g/dl during their pregnancies, the non-availability of Rh_{null} blood donors in South Africa, Susannah's excellent clinical state and our concern for the condition of her infant at birth, a unit (475 ml) of blood was withdrawn from Susannah with her consent both at 20 and 27 weeks of pregnancy for storage in liquid nitrogen. This blood loss was well tolerated. Although the amount of blood lost by her at delivery and subsequent sterilization was minimal, one of these units was recovered from storage at this time and returned to her. The successful recovery of more than 80% of the cells during the reconstitution showed that the osmotic fragility of her cells was not increased significantly. No complications were noted and 7 days later, Susannah was discharged from hospital.

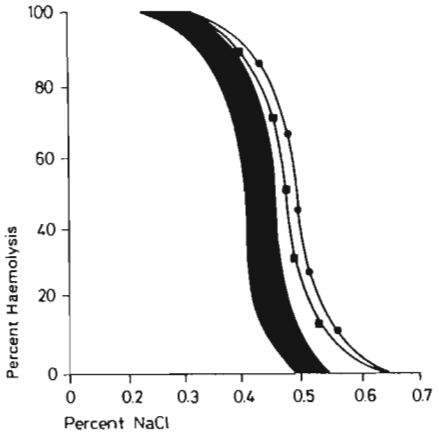

Fig. 2. Osmotic fragility curve for Susannah F. red cells showing the essentially similar results obtained during her pregnancy ■ and at 4 months post partum (●). Shaded area includes all results for red cells of 5 normal blood donors matched with Susannah F. for age and sex.

The Infant

The anti-Rh29 serum antibody quantitation and titration results obtained at intervals during Susannah's pregnancy and after her delivery were initially low (microscopic titre 4) and showed no significant changes during the ante-natal period. At 37+ weeks, the amniotic fluid analysis reading at 450 nm was 1.107 and lecithin-sphingomyelin ratio 2.6. The 450 nm reading, which showed a point situated in the upper middle zone on *Liley's* chart [3], caused some concern and, at 38 weeks, Susannah was delivered of her infant by caesarian section. The infant, a female, weighed 2,800 g.

At birth, the infant had Apgar scores of 9/10 at 1 min, 10/10 at 5 min, and 10/10 at 10 min. The cord blood direct antiglobulin test was 1+ macroscopic positive, total serum bilirubin 135 µmol/l, haemoglobin (Hb) 16 g/dl, haematocrit (Hct) 0.47 l/l and blood group A, Rh_2rh (cDE/cde). Anti-Rh29, but not anti-A, antibodies were eluted from the cord cells. After 2 days, the serum bilirubin level had risen to 230 µmol/l, but it fell thereafter fairly steadily and at 10 days was 132.5 µmol/l. Blood transfusions were not considered necessary. At 4 months, the Hb was 14.9 g/dl, Hct 0.44 l/l, total serum bilirubin 31 µmol/l and direct antiglobulin test negative. The blood group of the infant was confirmed as A, Rh_2rh.

Discussion

Although the blood of Susannah F. exhibited all the characteristics associated with the Rh_{null} syndrome, no evidence was detected during her pregnancy of the normocytic, normochromic anaemia (Hb 8 g/dl)

reported by *Bar Shany* et al. [2] in 2 other pregnant Rh_{null} women. Moreover, Susannah maintained her Hb and Hct levels so well that she was able to donate two units of her blood for liquid nitrogen storage without any ill effects. This showed that Rh_{null} cells in a pregnant woman need not always be a cause for concern haematologically.

Since no prior information was available, Susannah's pregnancy was managed in the same way as that of an Rh-negative mother whose anti-Rh_o(D) antibodies were of similar concentration and titre to the anti-Rh29 antibodies in Susannah's plasma. Amniocentesis, although not indicated, was performed at 37 + weeks merely to confirm that the course of action adopted for Susannah had been correct. However, the optical density reading, in the upper middle zone of *Liley's* [3] chart, immediately raised doubt, and Susannah's infant was delivered forthwith. The doubt proved unfounded when her infant was noted at birth to be suffering only mildly from haemolytic disease of the newborn. The result confirmed that amniocentesis, performed at this late stage in pregnancy, does not necessarily provide a reliable guide to an infant's condition, and we were consequently satisfied that Susannah's pregnancy had indeed been managed correctly.

Acknowledgements

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B.J. Gibbs, Technical Manager,
Blood Technology Division,
Western Province Blood Transfusion Service,
PO Box 3788,
8000, Cape Town (South Africa).

Paper 10

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A CASE OF HEREDITARY OVALOCYTOSIS

by P. P. MOORES and P. N. BUCK
The Natal Blood Transfusion Service, Durban

Introduction

Human genetic studies, particularly those concerned with linkage determinations, almost invariably involve the blood groups[1]. Until recently, the various blood groups were thought to be inherited independently of sex and of ability to taste certain substances, but it has since been shown[2], that the locus for the Xg blood group system gene is sex-linked. At one time it was thought that the blood groups were inherited independently of one another, but eventually it was shown [3, 4, 5], that there was apparent linkage between the Lewis and Lutheran loci; later demonstrated [6, 7, 8, 9], to be between the Lutheran and the secretor loci.

Many attempts have been made to demonstrate linkage between blood groups and other human characteristics. With two notable exceptions, however all have failed. A list of these studies is given by Race and Sanger.[10]

Linkage between a blood group locus and a non-blood group locus was first demonstrated in 1953 [11, 12, 13, 14], in the linkage of a Rhesus gene and that for oval red cells: ovalocytosis or elliptocytosis. Some workers distinguish between these two expressions, apparently on the basis of the degree of elongation of the red cells, but in this article the two terms may be regarded as interchangeable.

It is believed[15] that the evidence for ovalocytosis/Rhesus linkage is overwhelming, but that nevertheless ovalocytosis depends on either of two dominant genes, the locus of one of them being linked to the Rhesus locus. A recent study[16] indicates a very low recombination frequency, thus proving that the linkage is even closer than had previously been suggested.

Two years after the demonstration of ovalocytosis/Rhesus linkage, linkage was found[17] to exist between the ABO locus and that carrying the gene for hereditary onycho-osteodysplasia (the rare "nail-patella syndrome").

The erythrocytes of non-mammalian vertebrates are characteristically oval and nucleated. Camel's erythrocytes are oval and non-nucleated.[18] In man, however, only occasional erythrocytes of non-anaemic persons are oval,[19] (between 1 and 15 per cent) although in many cases of macrocytic anaemia the figure is over 25 per cent. This is known as "symptomatic ovalocytosis". The average ovalocyte is 8.1 by 5.3 microns, but extremes of 12.2 by 1.6 microns are encountered.

The first reported case of 'hereditary ovalocytosis' was published in 1904 by Dresbach, and it has since been estimated[20] that one person in every 2,500 of the random population has the condition. Our own observations suggest that the South African frequency is much lower than that,

despite the belief that the incidence is higher among persons of Dutch, German and Italian descent. It has not been detected in the Hindu.[21]

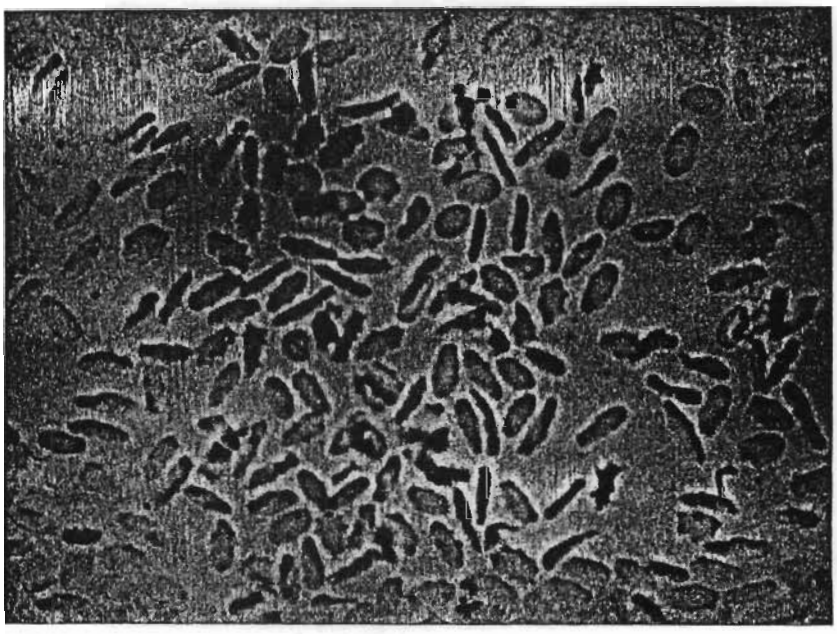
This paper presents a study of the presence of ovalocytosis in a South African family of Caucasoid descent.

Case History

On 5th February, 1963, the propositus, Miss D. M. Juckes donated blood for the second time. Miss Juckes is a 19-year-old person of Caucasoid descent, in good health, and is employed as a bank clerk.

In the course of routine pre-transfusion compatibility testing it was noticed (Fig. 1) that her erythrocytes were oval.

Figure 1



It was decided to investigate the family with a view to demonstrating

(*a*) whether or not linkage with the Rhesus locus was evident;

(*b*) if it was evident, to determine the particular gene at the Rhesus locus in this family;

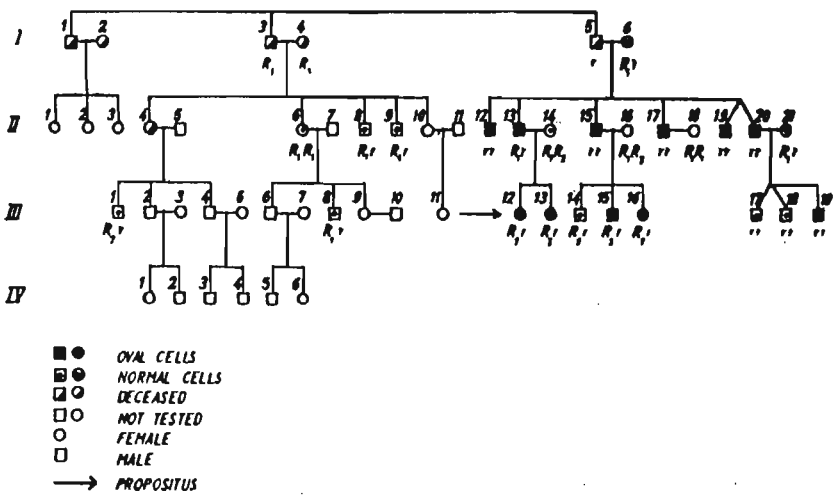
(*c*) and whether or not those members of the family who had ovalocytosis also showed evidence of haemolytic anaemia.

Method

The following investigations were performed with a minimum of delay on venous blood collected into ethylenediamine tetracetic acid, potassium salt.

1. Wet-preparation to demonstrate ovalocytes, if present.
2. Rhesus typing, using anti-C (rh′), anti-c (hr′), anti-D (Rh_0) including tests for D^u (Rh_0), anti-E (rh″) and anti-e (hr″).
3. Packed Cell Volume (P.C.V.) determination by the micro-haematocrit technique.[22]
4. Reticulocyte count by the brilliant cresyl blue technique.[23]

Figure 2



Result

To be read in conjunction with Figure 2.

TABLE 1

Line	*No.*	*Name*	*Age*	*ABO group*	*Probable Rh. genotype*	*Cell shape*	*Retics. %*	*P.C.V. %*
1	6	Christina	73	A	CDe/cde R^1r	Normal	0.9	43
2	6	Georgina	63	A	CDe/CDe R^1R^1	Normal	0.2	41
2	8	Charles	59	O	CDe/cde R^1r	Normal	0.8	45
2	9	Thomas	57	A	CDe/cde R^1r	Normal	0.4	43
2	12	Oswald	56	A	cde/cde rr	Oval	2.0	48
2	13	Ernest	49	A	CDe/cde R^1r	Oval	1.5	42
2	14	Hilda	49	A	CDe/cDE R^1R^2	Normal	0.2	41
2	15	Walter	46	A	cde/cde rr	Oval	0.8	38
2	16	Kathleen	45	A	CDe/cDE R^1R^2	Normal	0.5	33
2	17	Dennis	45	A	cde/cde rr	Oval	0.7	45
2	18	Beryl	37	B	CDe/CDe R^1R^1	Normal	0.3	43
2	19	Derrick	37	A	cde/cde rr	Oval	0.6	41
2	20	Desmond	37	A	cde/cde rr	Oval	1.0	40
2	21	Joan	34	O	CDe/cde R^1r	Normal	0.2	37
3	1	Raymond	42	A	cDE/cde R^2r	Normal	0.5	50
3	8	Peter	36	A	CDe/cde R^1r	Normal	0.4	45
3	12	Diana	19	A	cDE/cde R^2r	Oval	0.5	37
3	13	Colleen	13	A	cDE/cde R^2r	Oval	0.8	38
3	14	Ashley	16	A	cDE/cde R^2r	Normal	0.4	42
3	15	Nigel	13	A	cDE/cde R^2r	Oval	0.8	39
3	16	Hilary	9	A	cDE/cde R^2r	Oval	1.0	41
3	17	Alfred	13	A	cde/cde rr	Normal	0.2	37
3	18	Kenneth	13	A	cde/cde rr	Normal	0.2	35
3	19	Ronald	6	A	cde/cde rr	Oval	0.8	38

Discussion

It can be seen (Fig. 2) that the ovalocytosis gene is travelling on the same chromosome as one of the several cde (r) Rhesus units or genes in this family. There are no examples of crossing-over.

It is interesting that the series includes two sets of twins. One pair (uncles of the propositus) has insufficient evidence available here to show whether they are monozygotic or dizygotic: they are two of six brothers (no sisters) all of whom are group A and all but one of whom is cde/cde (rr). The father, unfortunately, is deceased.

The other pair of twins (cousins of the propositus) shows slightly more evidence of being monozygotic, but even so, this is slight, as the only sibling is a younger brother who could (if we disregard his age) equally well be a twin (same sex, ABO group and Rhesus type). The parents have only two different Rhesus units or genes among the four which jointly they possess and therefore the only appreciable differences apparent to it are that the father is group A and has oval cells, whereas the mother is group O and has normal cells. From this there seems to be a real chance that the twins are monozygotic as (in addition to the inconclusive evidence of the Rhesus types) they are of the same sex, and ABO group and have normal erythrocytes.

Ovalocytosis was absent from the blood of the paternal grandmother of the propositus and therefore, if mutation and illegitimacy are assumed to be absent, the responsible gene must have been present in the deceased paternal grandfather.

As all six children of the paternal grandparents of the propositus were affected, it seems safe to forget both mutation and illegitimacy, and it is tempting to suggest that the paternal grandfather may have been homozygous for the ovalocytosis gene. There are, however, two reasons which make this unlikely:

1. Each of the six has cde (r) at one Rhesus locus. If the paternal grandfather had been homozygous for ovalocytosis, his other Rhesus locus is unlikely to have carried another cde (r) and therefore there would have been fewer than the actual five out of six rr. It would be a truly remarkable coincidence if two of the very rare ovalocytosis genes not only met in one person (paternal grandfather) but *also* occurred on the same chromosome with an identical Rhesus unit or gene (r), particularly as with random distribution the chance of this occurring is only 1 in 3.14. This latter is because r occurs with r in 15.102 per cent of the Caucasoid population, but it occurs with other factors with the following frequency[10]:

R^1r	CDe/cde	31.676
R^2r	cDE/cde	10.966
R^0r	cDe/cde	1.995
$R^{1w}r$	C^wDe/cde	1.005
r′r	cdE/cde	.923
r″r	Cde/cde	.764
R^zr	CDE/cde	.189
Total	.. ..	47.519%

In our particular case, the probability that the paternal grandfather had something other than another r linked with his undoubted ovalgene is very slightly greater than 3.14 to 1, as at least one of the Rhesus genes or units of at least one of the two brothers of the paternal grandfather must have been R^1 (CDe).

2. If the paternal grandfather was a homozygote for the ovalocytosis gene then it must have been present in both of his parents (great-grandparents of the propositus). If we assume that neither of these great-grandparents were homozygous for the gene in question (a pretty safe assumption: homozygosity for a very rare gene in two different, non-inbred generations of the same family would really be something), then each of their three children (propositus's paternal grandfather and his two brothers) would have only a 1 in 4 chance of not possessing the gene. In at least one brother we know that one Rhesus gene or unit was R^1 (CDe), and judging by his children the ovalocytosis gene

was probably absent. So it becomes increasingly improbable that his other chromosome carried ovalocytosis either, judging again from the children (the propositus's father's cousins).

In this series the mean P.C.V. results were as follows (%):

	Oval Cells	*Normal Cells*
Male	41.4 (S.D. 3.3)	42.4 (S.D. 4.7)
Female	38.7 (S.D. 1.7)	39.6 (S.D. 3.6)

The difference of 1.0 per cent between the mean P.C.V. figures for the males with normal cells (42.4 per cent) and those with oval cells (41.4 per cent) is less than the standard error between the means (2.1 per cent) and therefore the fact that the mean for the males with oval cells is less than that for those with normal cells is not significant. Similarly, the difference of 0.9 per cent between the mean P.C.V. figures for the females with normal cells (39.6 per cent) and those with oval cells (38.7 per cent) is less than the standard error between the means (1.7 per cent) and therefore the fact that the mean for the females with oval cells is less than that for those with normal cells is again not significant.

The mean percentages of the erythrocytes which were reticulocytes were as follows:

Oval cells	0.95 (S.D. 0.42)
Normal cells	0.40 (S.D. 0.22)

The difference of 0.55 between these two mean reticulocyte percentages (0.95) and (0.40) is 3.87 times more than the standard error between the means (0.14) and this difference is significant.

There is evidence of increased blood destruction in 12 per cent of persons with the trait.[24] In this study we may conclude that judged by a statistically significant difference in the reticulocyte counts, there is evidence of increased erythrocyte destruction but this is so effectively compensated that there is no evidence of anaemia in the ovalocytosis bloods, as judged by the basence of a statistically significant difference in the P.C.V. percentages.

Summary

Ovalocytosis occurring in eleven members of a South African family of Caucasoid descent, was shown to be linked with the cde (r) Rhesus unit, and to cause increased erythrocyte destruction without anaemia.

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4. Dr. B. G. Grobbelaar, Medical Director of the Natal Blood Transfusion Service, for permission to publish, and for much helpful criticism.

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CHAPTER V

THE KELL SYSTEM

V.1 PAPERS

Informative family showing depression of Kell red cell antigens
Paper 28 by Moores, Daniels and Ford

Severe haemolytic disease of the newborn associated with anti-Js^b
Paper 34 by Lowe and Moores

V.2 INTRODUCTION

The antigen K (Kell) was discovered in 1946 by Coombs, Mourant and Race. Its antithetical partner, k (Cellano), was described in 1949 by Levine, Backer, Wigod and Ponder. In 1957, Allen and Lewis identified Kp^a, the first of the second pair of antigens in the Kell system, and Chown, Lewis and Kaita found the first example of K_o red cells (K_{null}). Kp^b, the antethetical partner of Kp^a, was discovered in 1958 by Allen, Lewis and Fudenberg. In 1965, Morton, Krieger, Steinberg and Rosenfield showed that Js^a (Sutter), identified by Giblett in 1958, and Js^b (Matthews), identified by Walker, Argall, Steane, Sasaki and Greenwalt in 1963, also belonged to this system. Anti-Ku, an antibody produced by people with rare K_o red cells which was specific for a very high frequency antigen, was described in 1961 by Corcoran, Allen, Lewis and Chown. Anti-KL, an antibody produced by people with rare McLeod red cells and specific for another very high frequency antigen, was described in 1968 by van der Hart, Szaloky and van Loghem. McLeod red cells express some Kell antigens weakly and are associated with chronic granulomatous disease. Anti-KL is a mixture of two antibodies, one being anti-Kx. It agglutinates K_o and most other but not McLeod red cells. Ul^a, a low frequency antigen found in 1968 by Furuhjelm, Nevanlinna, Nurkka, Gavin, Tippett, Gooch and Sanger, was shown by them to be part of the Kell system in 1969. The Kell antigens K11, discovered in 1971 by Guévin, Taliano and Waldmann, and K17, identified by Strange, Kenworthy, Webb and Giles in 1974, are probably antithetical.

The correct position in the system of the high frequency para-Kell antigens K12, K13, K14 and K18, all of which were identified between 1971 and 1975, is still uncertain [Issitt, 1985].

In 1952, Shapiro reported an example of anti-K and anti-Fy^a in a White male, and another of anti-k (Cellano) in a White female, in Johannesburg. The anti-k was only the third of this specificity recorded. In a nation-wide search, only five donors with compatible k- blood were found.

V.3 COMMENTARY

V.3.1 Attainments in Durban, 1961 to 1991

V.3.1.1 Heterozygous *K°*

Mr van Dev. was a regular blood donor. He had O Rh negative (dce/dce) red cells and both anti-CD and anti-Kp^b in his plasma [paper 28]. His red cells typed as Kp(a+b-) and, to the author's surprise, as K-k+^{w}, Js(a-b+^{w}) and Ku+^{w}. Some of his other Kell and para-Kell antigens were also expressed weakly, and more Kx antigen than usual was detected [quoted by kind permission of Blackwell Scientific Publications Ltd, Osney Mead, Oxford, England]. A family study was made. Mrs Ann Hoppe (Food and Drug Administration, Bethesda, USA) kindly helped collect blood samples from the members living in the United States. The family phenotypes in the Kell system strongly suggested that Mr van Dev. had the very rare genotype *$kKp^aJs^b/K°$*. An anti-k reagent known to demonstrate *k* dosage was used in meticulously-controlled titration studies with the family members' red cells. The titres required careful interpretation, however, as Ford, Knight and Smith had confirmed in 1977 that *K°* in *trans* to a gene complex in the Kell system which included *Kp^a* depressed the expression of the other Kell genes in that complex. In 1985, Issitt summarised the position in the Kell system as follows: *Kp^a* in *trans* or *cis* to *k* and *Js^b* sometimes but not always reduced k and Js^b antigen expression. *K°* in *trans* to a gene complex that included *Kp^a* reduced the expression of most, if not all, the Kell antigens

made. K° in *trans* to $kKp^{b}Js^{b}$ had no effect on the other Kell antigens in that complex, and no information was available on whether or not K° in *trans* to $KKp^{b}Js^{b}$ or $kKp^{b}Js^{a}$ affected those complexes. The authors concluded that the K° gene in Mr van Dev. had enabled them to see the extent to which his Kp^{a} gene had depressed his *k* and Js^{b} genes.

5.3.1.2 Haemolytic disease of the newborn

A Black Zezuru woman in Zimbabwe was found to have O, Le(a-b-) red cells with the rare Kell phenotype Js(a+b-) [paper 34]. She also had immune anti-A, anti-Le^{a} and anti-Js^{b} in her plasma. She had brought to the hospital her six-days-old infant, who had a septic umbilicus and was severely jaundiced. The infant's bilirubin level was found to be 16,5 mg/dl and haemoglobin level 7,8 g/dl; and the direct antiglobulin test was strongly positive. The infant's umbilicus responded well to antibiotics but his anaemia became progressively worse. At 4,6 g/dl of haemoglobin, no compatible blood donors having been discovered, the authors suggested obtaining a donation of blood from the infant's mother. This was done. As much plasma as possible was removed from the mother's donation, but no facilities were available for washing the red cells. The infant was given his mother's packed cells in a direct transfusion, and his subsequent recovery was uneventful. The strong positive direct antiglobulin test suggested that his mother's anti-Js^{b} had been the most serologically aggressive of her antibodies. Using her serum, diluted to avoid obtaining positive results with her anti-Le^{a}, the Js(b-) phenotype frequency in the Zezuru was later estimated to be approximately 1%. The authors encouraged others faced with a similar situation also to consider using the mother's blood. Provided that there was no ABO incompatibility between mother and child, it presented a practical alternative when compatible donor blood was not available.

Paper 28

P.P. Moores, G.L. Daniels and D.S. Ford. Natal Blood Transfusion Service, Durban, S. Africa, MRC Blood Group Unit, London, England and Commonwealth Serum Laboratories, Melbourne, Australia.

AN INFORMATIVE FAMILY SHOWING DEPRESSION OF KELL RED CELL ANTIGENS

A blood donor with anti-Kp^b in his serum was found to be K-kw, Kp(a+b-), Js(a-bw): his k, Js^b, Ku, K11 and para-Kell antigens are all clearly weakened. The mother of the propositus is K+kw, Kp(a+b+), Js(a-b+) and his father is dead.

Quantitative tests with many Kell antisera on the cells of the propositus and those of nine of his near relatives strongly suggest that the genotype of the propositus is $\underline{kKp^aJs^b}/\underline{K^o}$, and that the inheritance of $\underline{K^o}$ in *trans* permits the suppressing effect of $\underline{Kp^a}$ on its partner Kell antigens inherited in *cis* to be observed.

Abstract: 17th Congress of the International Society of Haematology and 15th Congress of the International Society of Blood Transfusion, 1978, p442.

An Informative Family showing Depression of Kell red cell Antigens

By P.P. Mastes, G.L. Daniels and D.S. Ford.

ISBT POSTER
Paris 1978

INTRODUCTION

IN THIS FAMILY, THE KELL PHENOTYPE OF THE PROPOSITUS, III-2, IS K-k+,Kp(a+b-), BUT HIS KELL GENOTYPE APPEARS TO BE $\underline{kKp^a}/\underline{K^o}$ AND NOT $\underline{kKp^a}/\underline{kKp^a}$ AS EXPECTED.

TABLE I SHOWS THE WEAK k, Js^b, Ku, K11 AND K12 ANTIGENS OF III-2 AND WEAK k ANTIGEN OF HIS MOTHER II-4, COMPARED WITH THE CONTROLS. NUMBERS OF REAGENTS WERE USED. THE Kx ANTIGEN OF III-2 IS ALSO SLIGHTLY INCREASED.

TABLE 1

RED CELLS	PHENOTYPE	KELL ANTIBODIES											PARA-KELL ANTIBODIES				
		K	k	Kp^a	Kp^b	Js^a	Js^b	Ku	KL	$U1^a$		Wk^a					Kx
		K1	K2	K3	K4	K6	K7	K5	K9	K10	K11	K17	K12	K13	K14	K18	K15
II-4	kK Kp(a+b+) Ge+	31	20	17	38	0	34	33	36	0	36	0	27	36	27	36	2
III-2	kk Kp(a+b-) Ge+	0	27	20	0	0	9	22	36	0	18	0	18	27	27	36	7
CONTROL 1	kk Kp(a-b+) Ge+	0	60	0	38	0	37	34	36								
CONTROL 2	kk Kp(a+b+) Ge+	0	62	19	38	0	39	34	36								
CONTROL 3	kK Kp(a-b+) Ge+	29	60	0	38	0	39	34	36	0	36	0	27	27	27	36	2

(SCORES BASED ON METHOD OF RACE AND SANGER)

TABLE II

RED CELLS	ANTI-k ROSSNER DOSAGE TITRATIONS 4	8	16	32	64	128	256	SCORE	PROBABLE GENOTYPE
II-4	8	5	-	-	-	-	-	13	$\underline{kKp^a}/\underline{KKp^b}$
II-2	10	8	1	-	-	-	-	19	$\underline{kKp^a}/\underline{KKp^b}$
II-1	5	3	-	-	-	-	-	8	$\underline{kKp^a}/\underline{KKp^b}$
III-2	10	5	-	-	-	-	-	15	$\underline{kKp^a}/?\underline{K^o}$
M.B.	10	5	1	-	-	-	-	16	$\underline{kKp^a}/\underline{K^o}$
CONTROL 1	10	10	10	8	3	-	-	41	$\underline{kKp^a}/\underline{kKp^a}$
III-3	10	10	10	10	5	-	-	45	$\underline{kKp^a}/\underline{kKp^b}$
I-1	10	10	10	10	8	5	-	53	$\underline{kKp^a}/\underline{kKp^b}$
CONTROL 11	-	-	-	-	-	-	-	0	$\underline{K^o}/\underline{K^o}$
II-6	10	10	10	8	3	-	-	41	$\underline{kKp^b}/?\underline{kKp^b}$
II-3	10	10	10	10	5	-	-	45	$\underline{kKp^b}/\underline{KKp^b}$
CONTROL 111	10	10	10	10	8	3	-	51	$\underline{kKp^b}/\underline{kKp^b}$

FIGURE 1

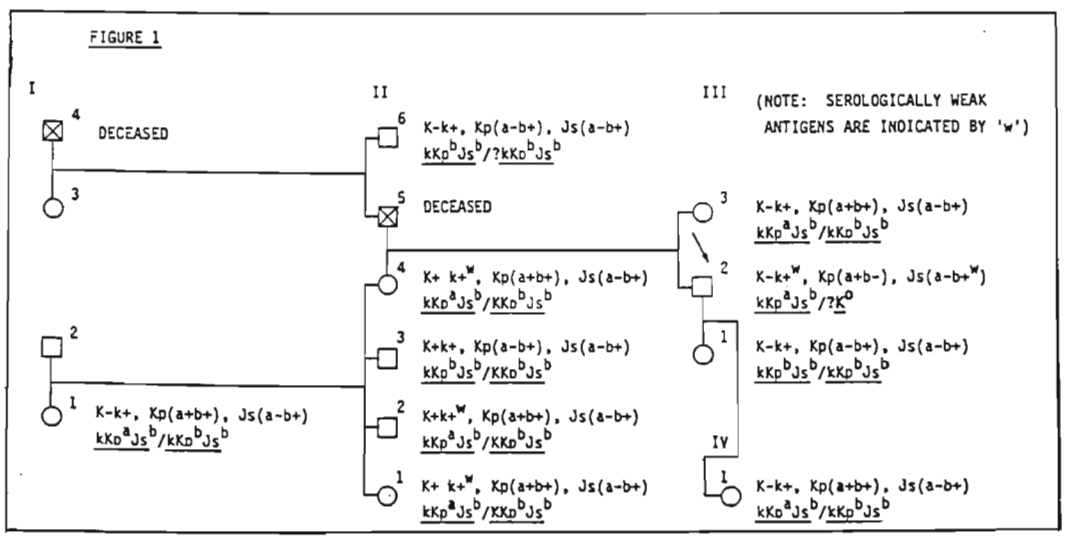

TABLE II SHOWS THE LOW SCORE OF III 2 RED CELLS WITH THE SPECIAL ANTI-k ROSSNER (FORD, KNIGHT AND SMITH, VOX SANG. 32, 220; 1977) WHICH PREVIOUSLY DETECTED $\underline{K^o}$ HETEROZYGOTES IN A NEW ZEALAND FAMILY. M.B. IS A KNOWN $\underline{K^o}$ HETEROZYGOTE FROM THIS FAMILY.

THE FAMILY STUDY IN FIGURE 1 INDICATES THAT THE PROBABLE $\underline{K^o}$ ALLELE WAS ALMOST CERTAINLY INHERITED THROUGH THE PATERNAL RELATIVES OF THE PROPOSITUS.

Another depressed Kell phenotype

The Kell groups of a healthy Sheffield donor, Mrs Elsie J., have been studied since 1959, by the late Dr Dunsford, Dr Darnborough and by our Unit. Her phenotype (Table 52) is

K–kw Kp(a+b–) Js(a–bw) Ul(a–) Ku(w)

Apart from a difference in the Kp phenotype her cells differ from those of the McLeod and Claas type only in that they react positively with anti-KL (with which the cells of McLeod and Claas do not react). As judged by limited tests, a sister of Mrs Elsie J. appeared to be of the same phenotype.

The cells of a maker of anti-Kp^b, Mr v. Dev., sent to us from Durban by Miss Phyllis Moores gave almost identical reactions, though the weakness of the antigens was not quite so marked in a repeat sample.

Blood Groups in Man.
by R.R. Race and R. Sanger; 6th edition, Blackwell Scientific Publications, Oxford, 1975, p298.

Paper 34

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Severe Hemolytic Disease of the Newborn Associated with Anti-Js^b

R. F. LOWE, A. T. MUSENGEZI, AND P. MOORES

From the Harari Central Hospital, Salisbury, and the Natal Blood Transfusion Service, Durban, South Africa

The red blood cells of the group A infant of a group O central African Negro woman of the Zezuru tribe with anti-Le^a and anti-Js^b in her serum were found to be strongly agglutinated by a commercial antiglobulin reagent six days after birth. The high concentration of bilirubin in the infant's serum gradually decreased but he became profoundly anemic and, in the absence of a suitable donor, was successfully transfused with blood from his mother. Subsequent tests using the maternal anti-Js^b diluted one in ten to avoid agglutination by the anti-Le^a showed that the frequency of the Js(b−) phenotype in group O Zezuru is approximately one per cent.

ANTI-Js^b (anti-K7), the antibody specific for Js^b (k7, an antigen in the Kell blood group system),[4] was first implicated as having been the cause of mild hemolytic disease of the newborn by Wake *et al.*[5] This report describes the infant son of a central African Negro woman, shown to have anti-Js^b in her serum, who was severely affected by hemolytic disease of the newborn. As compatible blood was not immediately available, the infant was transfused with blood donated by his mother, and he made a good recovery.

Case Report

When first seen the infant was six days old with a weight of 2.26 kg, a septic umbilicus, and a history of deepening jaundice. He was referred to Harari Central Hospital where tests showed that his serum bilirubin was 16.5 mg/dl, hemoglobin 7.8 g/dl, red blood cell count 2.31 million/μl, white blood cell count 6,400 per μl, platelet count 524,000 per μl, alkaline phosphatase 19 King-Armstrong units/dl, serum glutamic oxalic transaminase 6 units/liter and serum glutamic pyruvic transaminase 4 units/liter. The VDRL test was negative and the direct antiglobulin test with a commercial reagent was strongly positive.

The umbilical sepsis was successfully treated with antibiotics and during the next two days the infant's spleen and liver became palpable and his serum bilirubin fell to 13.0 mg/dl. By the fourth day (he was now ten days old) his hemoglobin had fallen to 4.6 g/dl. The mother was blood group O ccDEe. By indirect antiglobulin tests her serum agglutinated red blood cells of 66 group O central African Negroes, 40 group O Whites and all ten samples of a commercial red blood cell antibody identification panel. The infant's blood group was A ccDEe. It was decided to transfuse him without delay with red blood cells from his mother, removing as much as possible of the plasma beforehand. Approximately 40 ml of red blood cells were given, and on the following day the infant's hemoglobin had risen to 9.8 g/dl. Five days later his hemoglobin was 8.1 g/dl, red blood cell count 3.26 million/μl and no further treatment was considered necessary. He was discharged and when seen two months later looked well, his weight had increased to 4.65 kg. His hemoglobin on this occasion was 7.8 g/dl, red blood cell count 3.43 million/μl, alkaline phosphatase 4 King-Armstrong units/dl and serum bilirubin 0.8 mg/dl. At one year of age the infant was seen again and was completely well with no clinical signs of anemia.

Mrs. Anna, the infant's mother, was 30 years of age and gravida 6 para 5. She was a member of the Zezuru tribe of Central African Negroes from the Salisbury area. Her fourth pregnancy had terminated at six months in an abortion but there was no evidence that her first, second, third or fifth infants had suffered from hemolytic disease at birth. Her second and third infants had died in childhood aged 30 months and 13 months respectively. A sample of her blood was sent to Durban where one of us (P.M.) grouped her red blood cells as follows: O, MsU, P_1, ccDEe, hr^s+,

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hr^B+, C^w−, Lu(a−b+), K−k+, Kp(a−b+), Js(a+b−), Le(a−b−), Fy(a−), Fy:−3, Jk(b+), I+, Sd(a+), Co(a+b−), Di(b+), Ge+, Gy(a+), Vel+, Yt(a+), Sm+, At(a+), Yk(a+), Lan+, and Jr+.

Mrs. Anna's direct antiglobulin test was negative. Anti-A hemolysin was present in her serum and it was not inhibited by 2-mercaptoethanol. Her anti-A also agglutinated A_1 cells in saline and in enzyme titrations at 22 C to a titer of 256 and at 37 C to a titer of 512. In addition two antibodies, anti-Js^b and anti-Le^a, were identified in her serum and confirmed by absorption and elution studies. The anti-Le^a agglutinated Le(a+) Js(a+b−) red blood cells moderately well (++) by saline and enzyme techniques at 22 C but not by the indirect antiglobulin test. It was inhibited by 2-mercaptoethanol. The titer of her anti-Js^b, which agglutinated Le(a−) Js(a−b+) red blood cells weakly by enzyme techniques (+) but strongly by indirect antiglobulin test (++++), was 64 both at delivery and in another specimen drawn two months later. This antibody was not inhibited by 2-mercaptoethanol. Reference samples of group O Le(a−) K_0 and Le(a−) Js(a+b−) red blood cells kindly sent by blood grouping laboratories in the USA were not agglutinated by her serum on each occasion, excluding anti-N, -S, -C, -Lu^a, -Le^b, -Fy^a, -Fy^b, and Jk^a. Anti-K and anti-Kp^a were not excluded. Unfortunately an eluate could not be made from the first sample of the red blood cells of Mrs. Anna's infant as the sample was delayed on the journey to Durban and arrived totally hemolysed. However, in a further blood sample drawn from her infant at two years of age the red blood cell groups were found to be as follows: A_1, MNs, P_1, ccDEe, hr^s+, hr^B+, C^w−, Lu(a−), K−, Kp(a−), Js(a+b+), Le(a−b−), Fy(a−), Fy:−3, Jk(b+), I+, and Sd(a−).

Discussion

In the absence of an eluate which might have provided direct evidence of the identity of the antibodies attached to the red blood cells of Mrs. Anna's infant the strongly positive direct antiglobulin test six days after birth was believed to indicate that the most likely cause of the infant's jaundice and anemia was Mrs. Anna's anti-Js^b. However, in the case reported by Wake *et al.*[5] in which anti-Js^b was present, jaundice and severe anemia did not develop and this may indicate that Mrs. Anna's infant was far more severely affected because of the presence of both immune anti-A and anti-Js^b. It is unlikely that Mrs. Anna's immune anti-A alone was to blame for her infant's severe condition as his red blood cells were clearly well coated with immune globulin, and in hemolytic disease of the newborn due to anti-A and/or anti-B the direct antiglobulin test with normal broad-spectrum antiglobulin reagents is usually negative or only weakly positive.

Red blood cells from Mrs. Anna were used for her infant's blood transfusion as it was established that her red blood cells were not agglutinated by her own antibody (her direct antiglobulin test was negative) and she was the only person with serologically compatible red blood cells available when it was decided that her infant needed blood urgently. Fortunately she was group O, and further harm to her infant's red blood cells was minimized by removing as much plasma as possible before the transfusion was given. A better procedure might have been to have washed the cells free of plasma beforehand, but the hospital did not have the necessary equipment for this. It is hoped that the successful outcome may encourage others faced with a similar problem to consider using maternal antibody-compatible red blood cells, in instances where the ABO and Rh groups of mother and infant are compatible, before resorting to antibody-incompatible red blood cells from other persons.

Lowe,[2] using anti-Js^a only, found five Js(a+) individuals in 35 Zezuru tested. This corresponds to a Js^a phenotype frequency of 7.42 per cent which does not differ significantly from the Js^a frequency (9.45 and 4.17 per cent respectively) established by Fraser *et al.*[1,3] in 100 and in 98 mixed Bantu respectively in nearby Zaire (Congo). Using Mrs. Anna's serum diluted 1 in 10 with saline, which at this dilution was shown not to agglutinate Le(a+) Js(b−) cells, and in which no other antibodies (excluding anti-K and anti-Kp^a) were detected, three Js(b−)

individuals were found in 305 random group O Zezuru tested, giving a Js(b−) phenotype frequency in them of approximately one per cent. The gene frequencies in the Zezuru kindly calculated for us from this data are: $Js^a = 0.0990$, and $Js^b = 0.9010$.

Acknowledgments

We would like to thank the Secretary for Health, Rhodesia, for permission to publish this case, and Dr. C. Giles, Blood Group Reference Laboratory, London, for kindly confirming the identity of the anti-Js^b.

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Dr. R. F. Lowe, MRCS., LRCP., DCP., DTM & H., Senior Pathologist, Harari Central Hospital Laboratory, Salisbury, Rhodesia.

Dr. A. T. Musengezi, MB., ChB., DCH., Registrar, Department of Pediatrics and Child Health, University of Rhodesia, Salisbury, Rhodesia.

Miss Phyllis Moores, M.Sc., Nat. Dip. Med. Lab. Tech. S.A., Scientific Officer, Natal Blood Transfusion Service, 149 Prince Street, Durban 4001, South Africa.

CHAPTER VI

THE LEWIS SYSTEM AND SECRETION

VI.1 PAPERS AND STUDIES

Lewis groups and secretor status in Natal Bantu
Paper 14 by Moores and Brain

Observations on the Lewis red cell group Le(a+b+)
Unpublished study 9, M.Sc. thesis, p166-177

Association of the human Lewis blood group Le(a-b-c-d-) with the failure of expression of α-3-L fucosyltransferase
Paper 40 by Greenwell, Johnson, Edwards, Reed, Moores, Bird, Graham and Watkins

The secretion of A,B,H and Lewis blood group substances in the gastric juice and saliva of Chacma baboons (*Papio ursinus, Kerr*) and Vervet monkeys (*Cercopithecus Pygerythrus, Cuvier*)
Paper 24 by Downing, Moores, Bolstridge, Schleyer, Klomfass and Davidson

VI.2 INTRODUCTION

The first Lewis antibody, anti-Le^a, was discovered in 1946 by Mourant. In 1948, Andresen described anti-Le^b. Three Lewis phenotypes occur commonly. They are Le(a+b-), Le(a-b+) and Le(a-b-). Anti-Le^c was made in animals immunised with Le(a-b-) red cells. It reacts with the red cells of non-secretors of ABH substances who have Le(a-b-) phenotypes and has since been found in a human serum. Anti-Le^d was made in animals immunised with the red cells of secretors of ABH substances who had Le(a-b-) phenotypes. A fourth Lewis phenotype, Le(a+b+), is characteristic of infants of from a few weeks to about two years of age and is very rare in adults.

The Lewis antigens are now known to be primarily antigens of the tissues, rather than of the red cells [Race and Sanger, 1975, p323-349]. When an *Le* gene is inherited, the tissues provide a transferase which results in the production of Le^a and Le^b antigens: Le^a by people with an *se* (non-secretor) gene and Le^b by those with an *Se* (secretor) and an *H* gene. The *le* gene is silent. Cutbush, Giblett and Mollison [1956] found

that four samples of Le(a-b+) red cells were rapidly eliminated in a serum containing anti-Le^a. This confirmed that these cells had some Le^a antigen. In some twin chimeras with two populations of red cells, both populations have the same Lewis phenotype, regardless of the Lewis genes of the donor twin who supplied the host twin with cells from his erythropoietic tissue. Issitt [1985, p185] suggests that the probable explanation for Le(a+b+) red cells in adults is that the *Le* gene transferase competes more successfully than usual with the transferases provided by the *H* and *Se* genes.

VI.3 COMMENTARY

VI.3.1 Attainments in Durban, 1961 to 1991

VI.3.1.1 Lewis phenotype frequencies

By 1968, no Blacks in South Africa appeared to have been tested for their Lewis or secretor phenotypes. The authors obtained samples of blood and saliva from 181 Black donors and a number of White donor controls and published their results in paper 14. Four anti-Le^a and seven anti-Le^b reagents were used. As the anti-Le^b were inhibited by H substance, they were all type anti-Le^{bH} (no evidence that they merely had anti-H specificity was found). Reagents from Black, White and Indian donors were included, and they were all known to give the same results in all three populations. The salivas were tested with anti-A, anti-B and *Ulex europaeus* anti-H lectin. The findings showed that 57% of the Black donors had Le(a-b+), 22% had Le(a+b-) and 23% had Le(a-b-) red cells. As the donors with A_1 Le(b+) and B Le(b+) red cells numbered only slightly less than those with A_2 Le(b+) and O Le(b+) red cells, they were not excluded. The Le(a+b-) donors were all non-secretors, and the Le(a-b+) donors were all secretors, of ABH substances. Twenty-seven percent of the 181 Blacks were non-secretors of ABH substances, and 25% of 171 of them were non-secretors of Le^a substances. Among the Blacks with Le(a-b-) red cells, 20% were non-secretors of ABH substances. In the Blacks with group O red cells who secreted ABH substances, no correlation was seen between the amount of H on their red cells and in their saliva. The Black donors secreted as much H antigen in their saliva as

the White donors. Some donors had ABH and Lewis substances in their sera but not in their salivas, and some had abundant ABH substances in their salivas and none in their sera. This finding was thought not to confirm the "overflow" theory [Vos and Comley, 1967; Race and Sanger, 3rd ed., 1958, p204 and 209] whereby, when sufficient Lewis substance is secreted in the saliva, it "spills over" into the serum.

VI.3.1.2 The Le(a+b+) phenotype

The results of a small survey made in 1971 by Dr G.H. Vos (Natal Institute of Immunology, Durban) in collaboration with Dr Ph. Sturgeon (UCLA Medical School, Los Angeles, USA) had suggested that 6% of Natal Blacks who secreted Le^b substance in their saliva, secreted more Le^a substance than expected and might have Le(a+b+) red cells. In response to this, the author tested the red cells of 422 group O Black and 257 group O White blood donors with three examples of anti-Le^a, four of anti-Le^{bL} and two of anti-Le^x [Study 9]. The reagents were all known to agglutinate a sample of Le(a+b+) red cells from another source. They were used both unabsorbed and after absorption according to the advice of Sturgeon and Arcilla [1970]. The absorptions were first made with enzyme-treated group O Le(a-b-) red cells to remove anti-H. The anti-Le^a reagents were then re-absorbed with enzyme-treated Le(a-b+), and the anti-Le^{bL} reagents with enzyme-treated Le(a+b-), red cells. The findings showed that the red cells of 19,9% of the Black, and 15,2% of the White, donors had been agglutinated by all three unabsorbed reagents. Nine of the Black donor samples were selected for further study. The absorbed anti-Le^{bL} and anti-Le^x reagents agglutinated them all strongly but the absorbed anti-Le^a reagents agglutinated them either weakly or not at all. As the absorbed reagents had also agglutinated the Le(a+b+) control cells, the author suggested that the unabsorbed anti-Le^a had contained some anti-Le^b. The author's Lewis antigen frequency studies showed that all of the Le(a+b+) Blacks, and approximately 40% of the Le(a+b+) Whites, should have been included with the Le(a-b+) donors in their respective races. The remaining Le(a+b+) Whites should have been included with

the Le(a+b-) White donors. No true Le(a+b+) red cells had been detected. The false nature of the Le(a+b+) phenotype was seen when the absorbed anti-Le^a reagents gave weak or negative results. Study 9 confirmed that Le^a and Le^b were both present on Le(a-b+) red cells.

VI.3.1.3 The Le(a-b-c-d-) phenotype

In paper 40, the authors described two unrelated persons who lacked the very common enzyme α-3-L-fucosyltransferase, which catalyses the transfer of L-fucose to the O-3 position of N-acetylglucosamine. The persons' red cells grouped as Le(a-b-c-d-), and no Le^a, Le^b, Le^c and Le^d substances were detected in their saliva. Neither condition had been recorded before but evidently they were connected. Both persons were Blacks; one was referred from Durban because a sibling was a dispermic chimaera and the other was from the United States. No more examples were identified in 676 further Blacks or 61 Whites from Durban whose blood samples were Lewis-typed there in full, or in 1366 further persons from the United States.

VI.3.1.4 Lewis substances in animal secretions

Samples of saliva and gastric juice from 21 Chacma baboons and 23 Vervet monkeys were examined by agglutination-inhibition titration technique for secretion of A, B, H, Le^a and Le^b substances [paper 24]. Anti-A was used with group A_2 red cells to detect A substance, anti-B with group B red cells to detect B substance and anti-H lectin (*Ulex europaeus*) with group O red cells to detect H substance, all by saline technique at room temperature. Anti-Le^a was used with Le(a+b-) and anti-Le^b with Le(a-b+) red cells to detect Le^a and Le^b substance, respectively, by a one-stage ficin tile technique. Neither the anti-Le^a nor the anti-Le^b was inhibited by H substance. Some baboon and monkey salivas gave negative results, while with others the inhibition titres ranged from 2 to 2048. H substance was identified in all the baboon and monkey salivas. A substance was detected in 18 baboon and 21 monkey salivas and B substance in 11 baboon and 7 monkey salivas. Le^a substance

was not detected in any baboon salivas but it was identified in 21 monkey salivas. Le^b substance was detected in 13 baboon and 22 monkey salivas. With the gastric juice samples, H substance was detected in 17 baboons and 15 monkeys, A substance in 20 baboons and all the monkeys and B substance in 13 baboons and 9 monkeys. Le^a substance was detected in 5 baboons and 6 monkeys and Le^b substance in 8 baboons and 8 monkeys. One baboon had no A substance and 7 baboons no Le^b substance either in their saliva or their gastric juice. When present, B substance was detected both in the saliva and the gastric juice. In general, the A and B inhibition titres were higher in the baboons than in the monkeys, and the Le^b inhibition titres were higher in the monkeys than in the baboons. The findings suggested that the *H* and *Se* genes in these animals had independent activities.

Paper 14

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Lewis Groups and Secretor Status in Natal Bantu

P. MOORES AND P. BRAIN

From the Natal Blood Transfusion Service, Durban, Republic of South Africa

Of 181 South African Bantu subjects 27% were non-secretors of ABH substance, and 25% of 171 were non-secretors of Le^a substance; 54% had red cells of the Le(a—b+) phenotype, 22% Le(a+b—), and 23% Le(a—b—); 20% of this last group were non-secretors of ABH substances. Among secretors of group O there was no correlation between the amount of H antigen on the red cells and the amount of H substance secreted in the saliva. Some findings for ABO and Lewis substances in the serum are presented with reserve.

THE ABO blood groups and the H antigens of South African Bantu are remarkable in many respects,[3, 4] but to our knowledge no work has been published previously on their Lewis groups and secretor status.

Material

The Bantu examined were young adult volunteers of both sexes from a teachers' training college near Durban. Almost all were of the Zulu tribe. They were not selected for blood group, and as far as is known were unrelated. The Europeans used for comparison were a group of university students who attended a blood donor clinic on a particular day, together with a few older blood donors. They were selected for blood group, very few of group A having been called for the particular clinic concerned.

Reagents

Human sera used for ABO typing were chosen for their content of naturally occurring hemagglutinins and met the minimum requirements of the National Institutes of Health, U. S. Public Health Service. The anti-H and anti-A_1 were saline extracts of the seeds of *Ulex europeus* and *Dolichos biflorus* respectively.

Four anti-Le^a sera were used. One from a Bantu donor of group A and another from a European of group A_1 were used with bromelin for red cells of groups A_1, A_2 and O.[11] A third serum, from a Bantu donor of group B, had a saline agglutinin and was used for cells of groups B and O. A fourth serum from a European donor of group AB was used by the indirect antiglobulin method but only with AB cells.

There were seven anti-Le^b sera, obtained from Bantu, Indian and European donors. All but one, who was A_2B, were of group A. Four sera contained saline agglutinins and three were used with bromelin. All were inhibited by salivary H substance.

The salivary type of the donors of the anti-Lewis sera was unknown.

Methods

Red Cells

ABO grouping was performed in tubes at room temperature, centrifuging after one hour and reading with the naked eye. Cells classified as A_1 gave strong (4+ or 3+) agglutination with Dolichos reagent; all others of group A were classified as *other A* because of the known complexity of the A subgroups in the Bantu.[3]

Lewis grouping was performed in tubes at room temperature by a method appropriate to the serum, as previously mentioned.

Saliva

All salivas were boiled for ten minutes immediately after taking. Preliminary tests had shown that this method of boiling produced no more than 2% concentration of the saliva.

Anti-A (dilution 1/8), anti-B (1/8) and Ulex (1/4), already referred to, were used for secretor tests. An equal quantity of undiluted saliva was added, the tubes shaken, and the mixtures allowed to stand for 30 minutes at room temperature. The appropriate cells (A_2 for anti-A) were then added in 2% suspension and the tubes left a further hour at room temperature before reading. For Lewis testing an undiluted anti-Le^a serum was used with bromelin,[11] and an undiluted anti-Le^b in saline, performing the tests as above except that the temperature was 12 C and the tubes were centrifuged before

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TABLE 1. *Bantu—Distribution of Lewis and Secretor Phenotypes in Saliva and Red Cells*

Genotype	Saliva: Substances secreted ABH	Le^a	Le^b	Red Cells: Anti-Le^a	Anti-Le^b	ABO group	No. observed	Total
Se Se, LL	+	+	+			A_1	15	
						other A	12	
Se Se, Ll				−	+	B	14	
						A_1B	2	83
Se se, LL						other AB	3	
						O	37	
Se se, Ll				−	−	A_1	1	
						B	5	7
						A_1B	1	
Se Se, ll	+	−	+	−	+	A_1	2	
						other A	2	10
						B	3	
Se se, ll						O	3	
				−	−	A_1	9	
						other A	1	
						B	2	25
						other AB	1	
						O	12	
se se, LL	−	+	−	+	−	A_1	9	
						other A	9	
						B	7	38
						other AB	1	
						O	12	
se se, ll	−	−	−	−	−	A_1	3	
						other A	2	8
						O	3	
						TOTAL		171

reading. The same standard Le(a+b−) and Le(a−b+) cells were used throughout.

Serum

For these tests the standard anti-A was diluted 1/4, the anti-B and Ulex 1/8, and the Lewis antisera were used undiluted. The tests were performed exactly as for saliva, but often there was difficulty in interpreting the results, especially with anti-H reagent.

Results

Secretor Status

Saliva from 184 Bantu was available; one specimen was excluded for possible confusion of specimens and two for doubtful results in the test. Of the remaining 181, 49 (27%) were non-secretors, giving gene frequencies Se 0.48, se 0.52.

Secretion of ABH, Le^a and Le^b Substance in the Saliva, and Lewis Groups of Red Cells

Of the 181 Bantu subjects whose salivas were studied, four were excluded for doubtful results in saliva testing and six for incomplete (no anti-Le^b) grouping of the red cells. Results for the remaining 171 are shown in Table 1.

The distribution of the red cell Lewis phenotypes shows no significant inhomogeneity with respect to the four blood groups A_1, other A, B and O ($\chi^2 = 6.47$ for 6 d.f., $p \cong 0.4$).

The frequency of the saliva phenotype Le^a + is 0.75, giving gene frequencies L 0.50, l 0.50.

For comparison, a group of 74 Europeans was studied (Table 2). The Lewis grouping of the salivas in this sample could not be relied on because the subjects had been sucking candy which interfered with the tests.

Table 3 shows the frequencies of red cell Lewis phenotypes in the two groups.

Relation Between Anti-H Titer of Red Cells and H Substance in Saliva (Bantu O Secretors only)

The red cells of 29 Bantu O secretors were tested with four dilutions of Ulex reagent (¼, ⅛, 1/16, 1/32) and on the scores obtained, which varied from 1 to 12, the subjects were divided into two classes, one with high scores (14 subjects; mean score 8.8) and one with low scores (15 subjects; mean score 4.5). Saliva specimens were tested in six doubling dilutions, beginning at 1/16, for inhibition of the same Ulex reagent diluted ¼. The high-scoring group had a mean salivary inhibition level of 3.2 tubes and the low-scoring group a mean of 3.6 tubes.

ABO and Lewis Substances in the Serum

The findings in this section are presented with reserve, since it was difficult to get consistently reproducible results on successive specimens from the same donor.

Table 4 gives data on sera that did not contain the combination of substances expected from the Lewis grouping of the red cells [i.e., red cells Le (a+b−), all sera should contain Le^a but no Le^b substance; red cells Le (a−b+), all sera shoulld contain Le^b but no Le^a substance; red cells Le (a−b−), all sera should contain neither Le^a nor Le^b substance].

It was not uncommon for ABH, Le^a or Le^b substances to be detected in the serum when they were absent from the saliva. This was found in 7.3% of the 179 Bantu sera examined and in 19% of the 74 European ones, a difference that is significant at the conventional level. It was observed in non-secretors only (Table 5).

Discussion

As no other results from Bantu are available, we may compare our findings with those for West African and American Negroes.[2, 6, 10, 13]

The non-secretor phenotype frequency of 27% in the Bantu is very similar to the figure of 28% found in American Negroes,[10] but differs from the 38% observed in Lagos

TABLE 2. *Europeans—Distribution of ABH Secretor Phenotypes and Lewis Groups of Red Cells*

	Red Cells Anti-Le^a	Red Cells Anti-Le^b	ABO group	No. observed	Total
Secretors	—	+	A_1	4	51
			other A	1	
			B	13	
			A_1B	1	
			other AB	1	
			O	31	
Non-Secretors	—	—	A_1	1	5
			B	2	
			O	2	
	+	—	A_1	3	17
			other A	2	
			B	1	
			O	11	
	—	—	B	1	1
			TOTAL		74

TABLE 3. *Frequencies of Red Cell Lewis Phenotypes*

	No. examined	Le(a−b+)	Le(a+b−)	Le(a−b−)
Bantu	171	93 (54%)	38 (22%)	40 (23%)
European	74	51 (69%)	17 (23%)	6 (8%)

$\chi^2 = 7.70$ for 2 d.f., $p \approx 0.02$.

TABLE 4. *Unusual Combinations of Lewis Substances in the Serum*

Red cell type		No. examined	Serum No. observed	
			Le^a absent	*Le^b present*
Le (a+b−)	Bantu	38	4	2
	European	17	2	11
			Le^a present	*Le^b absent*
Le (a−b+)	Bantu	93	7	22
	European	51	—	1
			Le^a present	*Le^b present*
Le (a−b−)	Bantu	40	1	6
	European	6	—	6

TABLE 5. *Presence of Blood Group Substances in Serum when Absent from Saliva*

	Red cells Anti-Le^a	Red cells Anti-Le^b	Blood group	Substances present in serum, absent in saliva	No. times observed
Bantu Non-Secretors	+	—	A_1	A	2
			other A	H	3
			B	Le^b	1
			O	H	4
				H, Le^b	1
	—	—	A_1	A	1
			O	H	1
European Non-Secretors	+	—	A_1	A	1
				Le^b	1
			other A	H	1
			B	B, Le^b	1
			O	H	2
				H, Le^b	6
				Le^b	1
	—	—	B	Le^b	1

by Barnicot and Lawler.[2] However, there may be much tribal variation in non-secretor frequency among West Africans; for example, Lawler *et al.*[6] found 11% among the Fulani and 17% in the Habe tribe. A similar variation among American Negroes might account for the high figure of 39% found by Schiff[13] in 1940. It would be interesting to know whether there is much variation in secretor frequency among Bantu tribes.

Our non-secretor frequency of 27% is also near that observed by McConnell[9] in a large sample of Englishmen, but the distribution of the red cell Lewis phenotypes is different. The frequencies observed by us [Le (a—b+) 54%, Le (a+b—) 22%, Le (a—b—) 23%], are almost identical with those found in American Negroes by Miller *et al.*,[10] but differ from the proportions in Caucasians by virtue of a far higher frequency of the Le (a—b—) phenotype, some being non-secretors, and a corresponding lack of a—b+ subjects. This has been observed in West Africans[2] to an even greater degree [34% Le (a—b—)].

The frequency of the Le (a+) saliva phenotype is 75% in our Bantu, 54% in West African from Lagos,[2] 80% in the Fulani and 70% in the Habe[6] and more than 90% in Europeans.[12] This is another variable character that should be studied in other Bantu tribes.

If we ignore the Le (a—b—) phenotype, the Lewis grouping of the red cells gives a reliable indication of secretor status in the Bantu; all Le (a+b—) individuals observed were non-secretors and all Le (a—b+) were secretors. This does not hold for all populations, as witness the Australian aborigines reported by Vos and Comley.[14] It appears that the ratio of secreted Lewis to ABH substances in the Bantu is much the same as it is in Europeans; evidently this is not so in the interesting population of Vos and Comley.

It has been observed[5] that, although Le^b and H substances are not identical, some anti-Le^b reagents have a specificity similar to that of anti-H. Our anti-Le^b reagents, which are inhibited by salivary H substance, might better be described as anti-H

Le^b, or following Wiener[15] as anti-Le^H. But they are not identical with anti-H. Bantu of group O are remarkable[3, 4] for a very high level of red cell reactivity with plant anti-H substances, and one might therefore expect to find far more Bantu subjects of group O to have red cells positive with anti-Le^b than is observed in Europeans. We found that the cells of 60% of Bantu O subjects and 70% of Europeans react with anti-Le^b; the same figure of 70% was found by Grubb.[5] In our study, although the proportion of A_1 and B subjects whose cells react with anti-Le^b is slightly less than that of A_2 and O subjects, the difference is far from significant at the conventional level and we have not confined our anti-Le^b studies to bloods of group A_2 and O, as many authors have done. Nevertheless, it would be interesting to study Bantu samples of various groups, and especially of group A_1, with an anti-Le^b of the kind that does not react well with European A_1 blood. This is because Bantu of group A_1, unlike Europeans of that group, frequently have quite high red cell H scores. Such a study might throw further light on the cross-reactivity between anti-Le^b of the kind mentioned and anti-H reagents.

In this study the Lewis antisera were obtained from all three race groups in the donor population; but on the same specimens, of whatever race group, they all gave the same results.

Because of the known high H antigen level of the red cells in group O Bantu, we were interested to see whether the level of H substance in the saliva was also increased in these subjects. A comparison with a small number of European O secretor salivas showed that it is not increased much; a larger sample will be studied in the future. Within the Bantu group those with much H antigen on the red cells show no tendency to have more H in the saliva than those with little; the correlation, if there is one, may be in the opposite direction. This bears out the contention of Wiener and Kosofsky,[16] of Andresen,[1] and of Vos and Comley,[14] that the H antigens in saliva and on red cells are qualitatively different, perhaps even in a more fundamental way than a difference between water and alcohol soluble substances.

Our findings for serum are presented, despite their obvious deficiencies, because of their theoretical interest. It is commonly believed that if Lewis substance is secreted in sufficient quantity by organs such as the salivary glands, it spills into the serum; thus Vos and Comley[14] refer to the red cell Lewis groups as a *saliva cap*, and Race and Sanger,[12] say ". . . the saliva is saturated with Le^a substances" (i.e. in non-secretors) "and enough reaches the plasma to hook on to the red cells and make them give the Le (a+) reaction." But this cannot be so. Mäkelä and Mäkelä[8] showed that plasma lacking Le^b substance, to which salivary Le^b substance had been added, would not transform Le^b negative red cells to Le^b positive, whereas a plasma naturally containing Le^b substance would do so; and Levine and Celano[7] demonstrated that the same was true of Le^a substance, although tanned red cells would take up salivary Le^a. This suggests that there is a qualitative difference between the Lewis substances in saliva and in plasma, as there is a difference between H substance in saliva and on the red cells. Our own results suggest, but do not prove, that ABH and Lewis substances may sometimes appear in the serum when they are absent from the saliva. In non-secretors there may be, as is well known, a small amount of ABH substance in the saliva and this, though too small to be detectable in the saliva by our methods, may overflow into the serum and be detected there. But this cannot be so, because in many of our secretors, who have abundant ABH substance in the saliva, none was detected in the serum. If it is true that blood group

substances may appear in the serum when they are absent from the saliva and other secretions, the *overflow* theory in its simple form will have to be modified.

Acknowledgment

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Authors' address: Phyllis Moores, B.Sc., Principal Technologist, and Peter Brain, M.D., Deputy Medical Director, The Natal Blood Transfusion Service, P.O. Box 2356, Durban, Republic of South Africa.

Study 9

THE LEWIS GROUPS AND SECRETION

SECTION B

Personal Investigations

Observations on the Lewis Red Cell Group Le(a+b+)

Introduction

The Lewis red cell group Le(a+b+) is almost unknown in adults (Grubb and Morgan, 1949; Grubb, 1951; Simmons and Jakobowicz, 1951; Boettcher, 1965) but is characteristic in infants of from a few weeks to about two years of age (Andresen, Andersen, Jordal and Henningsen, 1950; Cutbush, Giblett and Mollison, 1956; Jordal, 1956; Lawler and Marshall, 1961; Mollison, 1972, p 252). However, Cutbush et al. (1956) realised unexpectedly that four adults previously grouped as Le(a-b+) were in fact Le(a+b+) when their cells were rapidly eliminated after being transfused into a patient who had potent circulating anti-Le^a; and Sturgeon and Arcilla (1970), using specially prepared and processed anti-Lewis reagents, described several adult Le(a+b+x+) members of a northern European and two Japanese families.

Claims have been made by Andresen (1948b); Grubb and Morgan (1949); Andresen et al. (1950); Grubb (1951); and Simmons and Jakobowicz (1951) that on occasions Le(a+b+) blood samples were demonstrated when cells were typed with potent agglutinating anti-Le^a, but Miller, Rosenfield, Vogel, Haber and Gibbel (1954) suggested this was probably due

to the presence of some anti-Le^b in the anti-Le^a reagent. Mollison (1972, p 250) also suggested that if suitable anti-Le^a sera were used by the antiglobulin technique most, if not all, group O and group A_2 (Le(a-b+) cells would appear to be Le(a+b+). The views of Miller et al. (1954) and of Mollison (1972) were supported by Vos and Comley (1967) who found 16% Australian Aborigines with red cells to some degree Le(a+) secreted H or AH substances. Vos and Comley (1967) concluded that in Aborigines it was not advisable to identify non-secretors of ABH substances by determining from tests on their red cells that their Lewis groups were Le(a+).

During a small survey initiated by Dr Vos in Natal In 1971, Dr Sturgeon found 6% Natal Negroes secreted Le^b substance and more Le^a substance than expected and suggested that their cells might be Le(a+b+). A study was undertaken as part of this thesis, using anti-Lewis reagents prepared (1) according to the specially stringent techniques of Sturgeon and Arcilla (1970), and (2) observing the normal precautions used routinely in Durban, to investigate the Lewis groups of the Natal Negroes more fully.

Materials and Methods

The blood samples were from group O Negro and White donors only and were prepared for use as indicated in Chapter II, special care being taken by washing the cells well with saline to remove all traces of residual serum from each sample to avoid inhibiting the reagents with serum H or Lewis substances in the cell suspensions during the test procedures.

The reagents used were three anti-Le^a, four anti-Le^b and two anti-Le^x from local Negro, Indian and White donors known to agglutinate red cells

well and to give clear specific results with the panel cells. They were all also shown to be capable of agglutinating a sample of Le(a+b+) cells from a member of the San race (a Bushman) gratefully received from Dr T. Jenkins, South African Institute of Medical Research, Johannesburg. The Lewis group of the San person was confirmed for Dr Jenkins by Dr R. Sanger, Blood Group Research Unit, Lister Institute, London. The specificity of the anti-Le^x reagents was confirmed by noting that both were able to agglutinate adult and cord cells equally well and in the same proportions (Jordal, 1956; Race and Sanger, 1968, p 320). None of the anti-Lewis reagents was inhibited by the saliva of a secretor of H but no Le^a or Le^b substances indicating that the anti-Le^b were all type anti-le^{bL} and not likely to be inhibited by traces of H substance during the test procedures.

The reagents were divided into two equal volumes: one volume being reserved for use unabsorbed without further treatment, and the other processed in accordance with the stringent methods advocated by Sturgeon and Arcilla (1970) as follows:

Preparation of cells for absorption

Packed, washed O Le(a-b-), O Le (a-b+) and O Le(a+b-) cells were treated with 0,25% ficin solution by mixing five volumes of each separately with one volume of the ficin solution for 10 minutes at ± 22°C (for preparation of 0,25% ficin solution, see Chapter II). After treatment the cells were washed again with saline three times and packed down, ready to be used for absorbing the reagents.

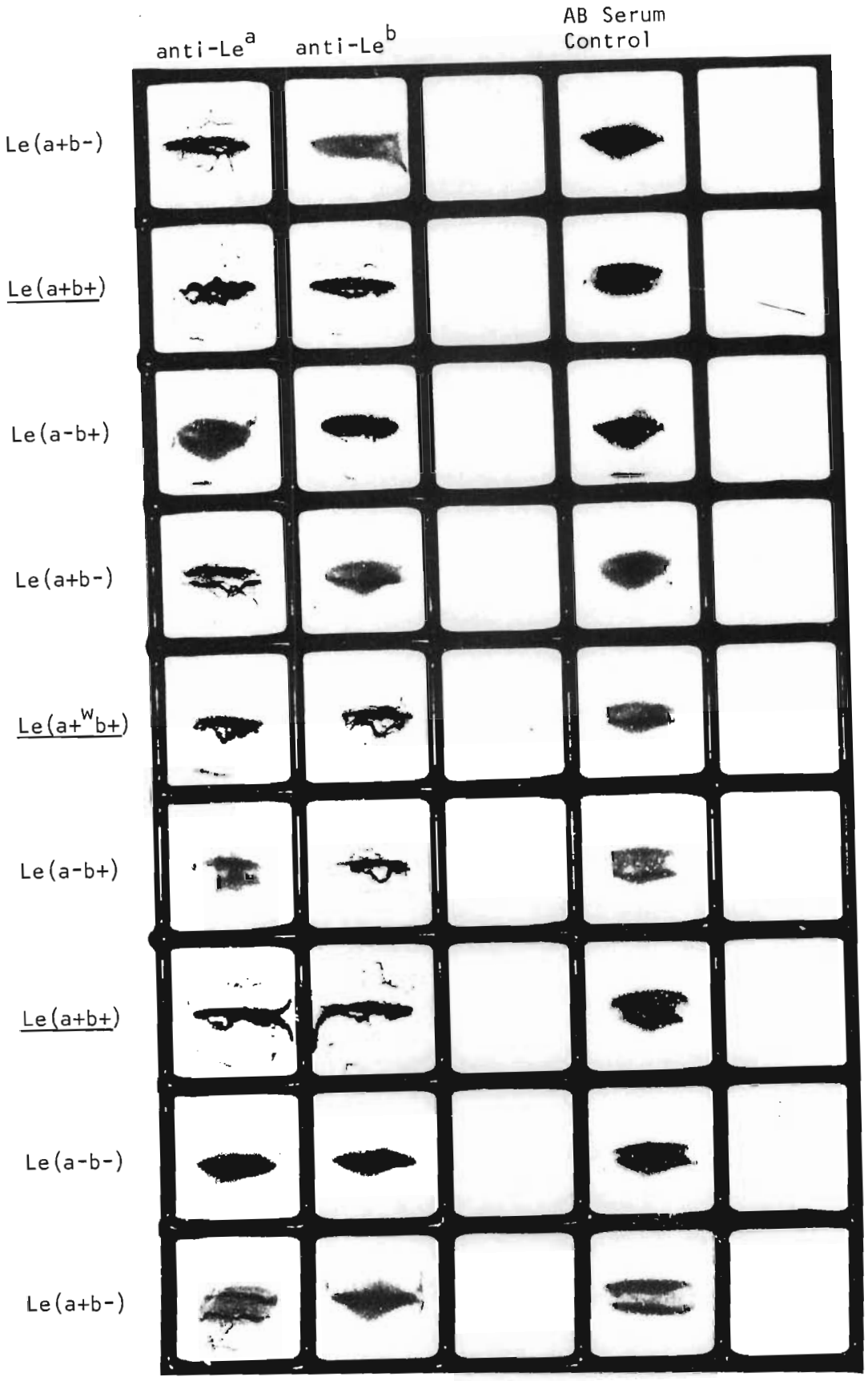

PLATE 3

Ficin Tile Test Showing Type Le(a+b+)
Red Cell Agglutination and Controls

Absorption of the reagents

All the reagents were first absorbed with equal volumes of ficin-treated O Le(a-b-) cells at $4^{o}C$ for 30 minutes to remove any anti-H present. The absorbed anti-Le^{a} reagents were then re-absorbed with ficin-treated Le(a-b+) cells and the absorbed anti-Le^{bL} reagents with ficin-treated Le(a+b-) cells at $4^{o}C$ for 30 minutes to remove any cross-reacting anti-Le^{b} or anti-Le^{a} respectively. On being tested subsequently with the panel cells, all the re-absorbed reagents were found to be still capable of agglutinating them strongly and specifically; but as a further precaution to ensure that the reagents were entirely specific, small aliquots of the re-absorbed anti-Le^{a} were absorbed again with equal quantities of ficin-treated Le(a+b-) cells, the re-absorbed anti-Le^{bL} with ficin-treated Le(a-b+) cells, and the absorbed anti-Le^{x} first with Le(a+b-) and then with Le(a-b+) ficin-treated cells. Following this, no agglutination of the panel cells was observed.

All the tests in this section were made by technique 6 (ficin tile test) and AB serum was always used as a neutral control serum with each test.

Results

The cells of 19,91% of the Negro and 15,18% of the White donors were agglutinated by unabsorbed anti-Le^{a}, anti-Le^{bL} and anti-Le^{x} and, as a result, were recorded as type Le(a+b+) (see Table IX.7). It was noticed, however, that although they were all agglutinated strongly (recorded agglutination value: 4) by unabsorbed anti-Le^{bL} and anti-Le^{x}, 80% of the Negro Le(a+b+) and 70% of the White Le(a+b+) samples were agglutinated strongly (recorded

TABLE IX.7

Lewis Blood Group Frequency in Negro and White Donors of Durban Showing that in Negroes Nearly all Le(a+b+) are Normally Included with Le(a-b+) Subjects but in Whites Approximately 60% of Le(a+b+) are Normally Included with Le(a+b-) and 40% with Le(a-b+) Subjects

Tribe or people tested	Area of habitation	Number tested	% Observed					% Expected		
			Le(a-b-)	Le(a+b-)	Le(a+b+)	*Le(a+^{w}b+)	Le(a-b+)	Le(a-b-)	Le(a+b-)	Le(a-b+)
Negro donors	Durban	422	22,04	23,22	(80% ⟷ 20%) 15,88 | 4,03 19,91		34,83 54,74	23,0	22,0	54,0
White donors	Durban	257	7,00	14,79	(70% ⟷ 30%) 21,46 ◂(6,67)—(4,23)▸ 71,55 10,90 | 4,28 15,18		63,04 67,32	6,0	22,0	72,0

* Denotes Le^{a} factor not as strong as in Le(a+b+) samples.

agglutination value: 4) by unabsorbed anti-Le^a and the remainder (20% Negroes and 30% Whites respectively) were only weakly agglutinated (recorded agglutination value: 2 or 1). Plate 3 shows the agglutination of the two types of Le(a+b+) observed compared with that of cells of other Lewis groups in a typical ficin tile test using unabsorbed anti-Le^a and anti-Le^{bL}, with AB serum included as a neutral control to detect evidence of auto-agglutination.

Nine cell samples from each race agglutinated strongly (recorded agglutination value: 4) by unabsorbed anti-Le^a, anti-Le^{bL} and anti-Le^x were selected for further study, but when these were tested subsequently with absorbed anti-Le^a, anti-Le^{bL} and anti-Le^x, it was found that although all of them were agglutinated strongly by absorbed anti-Le^{bL} and anti-Le^x, they were either only weakly agglutinated or not agglutinated at all by absorbed anti-Le^a (see Table IX.8). The cells of the Le(a+b+) San person were similarly strongly agglutinated by absorbed anti-Le^{bL} and anti-Le^x but not by absorbed anti-Le^a. It was therefore concluded that the 'Le(a+)' cell character detected in the tests with unabsorbed anti-Le^a in this study was probably incorrect and that the cells had been falsely agglutinated either due to the presence of a previously undetected antibody in the unabsorbed anti-Le^a reagents (the most likely undetected antibody was anti-Le^b, as suggested earlier by Miller et al., 1954) or, during the test procedure, Le^a but not Le^b antigen had somehow been 'lost' from the red cell membrane.

To examine the Lewis antigens of the red cell membrane more closely, the cells of further Le(a+b+) Negro and White donors, some known to be agglutinated strongly and others weakly by unabsorbed anti-Le^a, were subjected to treatment designed to remove or to destroy their Lewis antigens.

TABLE IX.8

Reactions of Le(a+b+) Negro, White and San (Bushman) Cells with anti-Lewis Reagents

Anti-Lewis Reagents	Red Cell Samples																				
	Le(a+b+) Negroes									Le(a+b+) Whites									Panel Cells			Le(a+b+)
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	Le(a+b-)	Le(a-b+)	Le(a-b-)	San (Bushman)
Unabsorbed																						
anti-Le^a M213	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	-	-	4
anti-Le^b PPM70	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	-	4	-	4
Absorbed																						
anti-Le^a M213	2	2	2	1	2	-	-	1	-	-	-	1	-	±	2	-	-	-	4	-	-	-
anti-Le^a M209	1	1	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-
anti-Le^a 89	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-
anti-Le^b PPM70	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	-	4	-	4
anti-Le^b M201	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4	4	-	4	-	4
anti-Le^b M146	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4	4	-	4	-	4
anti-Le^b PPM41	4	4	3	4	4	2	2	2	1	2	2	2	2	2	2	1	2	1	-	2	-	3
anti-Le^x M284	4	4	4	4	4	4	4	3	3	4	4	4	4	4	4	3	3	3	2	4	-	4
anti-Le^x M211	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	-	4

Some samples were washed six times with saline heated to 56°C, while others, washed three times with ± 20°C saline, were left overnight in saline at 4°C. In subsequent tests with unabsorbed anti-Le^a, anti-Le^{bL} and anti-Le^x however, the treated cells showed no loss of agglutinability. The H antigen strength of other strongly agglutinated Le(a+b+) cell samples (all group O, see earlier) was also compared in parallel titrations of Ulex anti-H reagent with the H antigen strength of normal group O Le(a+b-) and group O Le(a-b+) donor cells (Brain, 1966) but was not found to be increased. In titrations of unabsorbed anti-Le^a however, several strongly agglutinated Le(a+b+) cell samples were not agglutinated to the same titre as the control Le(a+b-) cells. Significantly, in addition, the sera of several Negro donors with strongly agglutinated Le(a+b+) cells were found to contain less Le^a substance than the sera of Le(a+b-) White donor controls but more than the sera of Le(a-b+) White donor controls.

The observed Lewis group frequency in the Negro and White donors was compared with the expected frequency (see Table IX.7) and it was seen that all of the Le(a+b+) Negro donors and approximately 40% of the Le(a+b+) White donors would probably normally be included with the Le(a-b+) members of their respective races while the remaining 60% of the Le(a+b+) White donors would probably normally be included with the Le(a+b-) White members in their race. Using a similar ficin tile technique Vos and Comley (1967) found 25 of 135 (18,5%) Australian Aborigine secretors compared with one of three Australian Aborigine non-secretors of ABH substances had Le(a+b+) cells, while only one of 171 (0,58%) White Australian secretors compared with five of 52 (9,6%) White Australian non-secretors of ABH substances had Le(a+b+) cells. Although the small number of Australian Aborigine non-

secretors tested made it difficult to assess the position in this race accurately, it was clear that in White Australians, as in the Natal White donors, a substantial proportion of the Le(a+b+) would normally be included with the Le(a+b-) samples.

Comment

The results in this section showed, using unabsorbed anti-Le^a, anti-Le^{bL} and anti-Le^x and technique 6 (ficin tile test), that it was possible to demonstrate an Le(a+b+) phenotype in both the Negro and White donors. However, when the anti-Lewis reagents were absorbed, only the anti-Le^{bL} and anti-Le^x continued to agglutinate the Le(a+b+) cells well although they were all shown to be still capable of agglutinating cells of other Lewis phenotypes satisfactorily.

Tests designed to inactivate or to destroy the Lewis antigens before the Le(a+b+) cells were exposed to agglutination by unabsorbed anti-Le^a, anti-Le^{bL} and anti-Le^x, although understood to be very effective, failed to do so, and this was believed to indicate that the cells were Le(a+b+) due to defects in the unabsorbed anti-Le^a and anti-Le^{bL} reagents rather than to adsorption by the cells of more than usual amounts of Le^a and/or Le^b substances from the donor's serum (as suggested by Dr Sturgeon). The possible adsorption of Le^b substance by the cells of some Le(a+b-) donors which might cause them to appear Le(a+b+) was also not acceptable, for according to the theory of Grubb and Ceppellini (1951), Le(a+b-) persons are genotype le.le and do not secrete Le^b substance, while Le(a-b-) persons do not secrete either Le^a or Le^b substances. Le(a-b+) persons, on the other hand, being genotype Le.Le or Le.le, may secrete both Le^a and Le^b substances, and it was logical to suppose that some of these donors might have secreted more than usual amounts of Le^a substance in their sera.

The results showed that the most likely reason for the apparent defect observed in the unabsorbed anti-Le^a and anti-Le^{bL} reagents in this study was undetected anti-Le^b in the anti-Le^a reagents and undetected anti-Le^a in the anti-Le^{bL} reagents. Authorities everywhere agree that both these antigens are probably present in differing proportions in the sera of most (suitably stimulated) Le(a-b-) persons but, while anti-Le^b is also sometimes made by Le(a+b-) people, few if any Le(a-b+) people make anti-Le^a as most of them secrete Le^a substance in their sera and may have Le^a substance adsorbed onto their red cells. Absorption of the anti-Le^a reagents with Le(a-b+) cells, either washed insufficiently beforehand to remove all traces of serum Le^a substance or having normal amounts of Le^a substance adsorbed onto the cells, will therefore successfully remove contaminating anti-Le^b, but will also weaken the anti-Le^a reagents to the point where although they may still be able to agglutinate Le(a+b-) cells, Le(a-b+) cells with more than usual amounts of adsorbed Le^a substance will no longer be agglutinated. Absorption of the anti-Le^{bL} reagents with Le(a+b-) cells, on the other hand, whether or not washed sufficiently and very unlikely to have adsorbed Le^b substance, will result in removal of contaminating anti-Le^a but will not be expected and was not shown to weaken these reagents. In support of this view the Le(a+b+) Negro donor cells were seen to be less strongly agglutinated by unabsorbed anti-Le^a than Le(a+b-) White control cells and the sera of the Le(a+b+) Negro donors contained more Le^a substance than the sera of the Le(a-b+) White controls but less than the sera of the Le(a+b-) White controls. It was perhaps unfortunate, but probably only by chance, that no Le(a+b-) sample masquerading as Le(a+b+) in the tests with unabsorbed anti-Le^a and anti-Le^{bL} was included among the nine Le(a+b+) White donor samples chosen for further study. The

presence of contaminating antibodies other than anti-Le^a and anti-Le^b in the reagents was considered eliminated by evidence that previously undetected anti-H was not responsible for the agglutination of some cell samples better than others, and by the finding that all the anti-Le^a reagents behaved in a similar manner when tested with the same cell samples.

The effect of an unusual technique such as the ficin tile test on the behaviour of the anti-Lewis reagents was considered partly to blame for the false Le(a+b+) phenotype detected in the Negro and White donors in this study, for it was known that 0,25% ficin solution was particularly effective in promoting agglutination by anti-Lewis and other cold agglutinins on a tile (personal observations). It was also observed that the frequency of this phenotype (19,9% in the Negro and 15,18% in the White donors) was considerably in excess of the 6% expected by Dr Sturgeon. Nevertheless some of the Le(a+b+) cell samples were agglutinated more strongly than others by unabsorbed anti-Le^a, and as it had been shown that the unabsorbed anti-Le^a reagents contained some anti-Le^b, this was considered to indicate that variable amounts of adsorbed Le^b rather than adsorbed Le^a antigen were present. The findings in this study therefore did not support the view of Dr Sturgeon that a true Le(a+b+) phenotype should be detectable in the Natal Negroes, but instead provided further evidence in support of the theory of Grubb and Ceppellini (1951) that it is probably not advisable to absorb certain anti-Le^a reagents with Le(a-b+) cells which may have adsorbed Le^a antigen.

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Association of the human Lewis blood group Le(a-b-c-d-) with the failure of expression of α-3-L fucosyltransferase

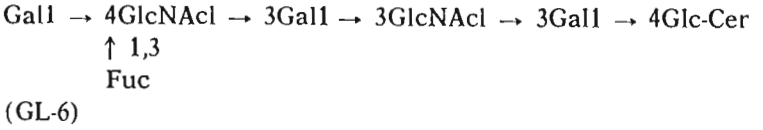
by P. Greenwell *, P.-H. Johnson *, J.-M. Edwards **, R.-M. Reed **, P.-P. Moores ***, A. Bird ***
H.-A. Graham ****, W.M. Watkins *

* Division of Immunochemical Genetics, MRC Clinical Research Centre, Harrow, Middlesex, U.K.
** Blood Bank, University of Texas Medical Branch, Galveston, Texas, U.S.A.
*** Natal Blood Transfusion Service, Durban, South Africa.
**** Ortho Diagnostic Systems Inc. Raritan, New Jersey, U.S.A.

THE Lewis blood group system [27] comprises two major antigenic determinants Le^a(Galβ1 → 3[Fucα1 → 4]GlcNAc) [28] and Le^b (Fucα1 → 2Galβ1 → 3[Fucα1 → 4]GlcNAc) [22] that are synthesised by the addition of α-L-*fucosyl* residues to the *O*-4 position of *N*-acetyl-**D**-glucosamine in, respectively, an unsubstituted Type 1 (Galβ1 → 3GlcNAc) precursor structure and a Type 1H (Fucα1 → 2Galβ1 → 3GlcNAc) structure [35]. Individuals grouped as Le(a — b+) on red cells are ABH secretors and those grouped as Le(a+ b —) are non-secretors [27]. The remaining individuals, grouped as Le(a — b —), lack both Le^a and Le^b antigenic activities. This group can be further subdivided according to whether they are ABH secretors

or non-secretors. In 1970 POTAPOV [26] described an antibody raised in a goat that after absorption with Le(a+ b —) red cells reacted with Le(a — b —) red cells from secretors but failed to react with Le(a — b —) red cells from non-secretors. This antibody was called anti-Le^d and the term anti-Le^c was reserved for the antibody which POTAPOV predicted would react specifically with an antigen on cells from Le(a — b —) non-secretors. Subsequently a human antibody was found [14] which did indeed react specifically with red cells from Le(a — b —) non-secretors and was accordingly termed anti-Le^c. Inhibition tests with defined oligosaccharides suggested that α-3-linked **L**-fucose attached to *N*-acetyl-**D**-glucosamine in a Type 2 (Galβ1 → 4GlcNAc) precursor chain was involved in the determinant structure detected by this antibody [14, 9]. By analogy with Le^b it was proposed that in the Le(a — b —) secretors Le^d specificity arose from the presence of **L**-fucose attached to the *O*-3 position of *N*-acetyl-**D**-glucosamine in a Type 2H structure (Fucα1 → 2-Galβ1 → 4GlcNAc) [14]. However, GRAHAM *et al.* [11] raised further anti-Le^c and anti-Le^d reagents in goats and, finding that anti-Le^a, Le^b, Le^c and Le^d were mutually exclusive in their reactions with red cells, proposed that the four structures detected by these antibodies were each built on Type 1 precursor chains. Subsequently inhibition of the anti-Le^d reagent with lacto-*N*-fucopentaose I (Fucα1 → 2Galβ1 → 3GlcNAc-β1 → 3Galβ1 → 4Glc) suggested that Le^d was in fact a Type 1H structure [12]. The unsubstituted Type 1 precursor structure was therefore proposed as the Le^c determinant [12] and support for this proposal came from the work of LE PENDU *et al.* [20], who showed that antibodies raised in rabbits with Galβ1-3GlcNAc-BSA as the immunogen, or isolated from anti sera raised against O Le(a— b—) saliva by absorption-elution on a column of the same synthetic disaccharide bound to silicate in place of BSA, agglutinated the red cells of Le(a — b —) non-secretor donors. Thus two different structures had been proposed for both Le^c and Le^d determinants (Table I).

More recently HANFLAND *et al.* [16] described two glycolipids isolated from the plasma of Le(a — b —) non-secretor donors which were characterised as: (GL-3)



and (GL-6)

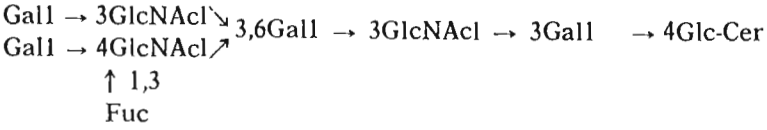


TABLE I

Structures proposed for Le^c and Le^d determinants.

SPECIFICITY	PROPOSED STRUCTURE	REFERENCE
Le^c	Galβ1 → 4GlcNAc... ↑ 1,3 Fucα	GUNSON and LATHAM [14]
	Galβ1 → 3GlcNAc...	GRAHAM *et al.* [12]
Le^d	Galβ1 → 4GlcNAc... ↑ 1,2 ↑ 1,3 Fucα Fucα	GUNSON and LATHAM [14]
	Galβ1 → 3GlcNAc... ↑ 1,2 Fucα	GRAHAM *et al.* [12]

GL-3 exhibited distinct, and GL-6 very strong, Le^c blood group activity against both human and goat anti-Le^c reagents. On the basis of these inhibition results, and other known factors relating to Le^c expression, the authors suggested that the branched structure,

Galβ1 → 3GlcNAcβ1↘
Galβ1 → 4GlcNAcβ1↗3,6Gal
↑ 1,3
Fucα

comprising 3-fucosyllactosamine (Galβ1 → 4[Fucα1 → 3]GlcNAc) in combination with a Type 1 oligosaccharide sequence and a 3,6-galactosyl branching point, constitutes the Le^c antigenic determinant [16]. Thus both the structures previously proposed (*Table I*) for the Le^c determinant [14, 12] are contained within the one oligosaccharide present in glycolipid GL-6.

Biosynthesis of such a fucosylated structure is dependent upon the activity of an α-L-fucosyltransferase that catalyses the transfer of L-fucose to the *O*-3 position of internal *N*-acetylglucosamine residues. An enzyme with this specificity was first described in human milk [32] and has subsequently been found in serum [30], saliva [37], mucosal tissues [4], red cells, lymphocytes, platelets [3] and granulocytes [13]. Expression of this enzyme is independent of Lewis blood group or secretor status [17, 18] and examination of large numbers of human sera at the Clinical Research Centre, U.K. had until recently failed to disclose any individuals who lacked α-3-L-fucosyltransferase activity. However, family studies have now revealed two, healthy, unrelated individuals who have no detectable α-3-L-fucosyltransferase activity in serum or saliva. Both these individuals also exhibited the unusual Lewis phenotype Le(a — b — c —d —).

MATERIALS AND METHODS

Substrates

GDP-L-[^{U14}]fucose (292 Ci/mol) was purchased from Amersham (U.K.). Phenyl β-D-galactopyranoside was obtained from Koch-Light (U.K.). 2'-Fucosyllactose (Fucα1 → 2Galβ1 → 4Glc), 3-fucosyllactose (Fucα1 → 3[Galβ1 → 4]Glc), lacto-*N*-tetraose (Galβ1 → 3GlcNAcβ1 → 3Galβ1 → 4Glc) and lacto-*N*-neotetraose (Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc) isolated from human milk as described [1], *N*-acetyllactosamine (Galβ1 → 4GlcNAc) prepared by a modification of the method of KUHN and KIRSCHENLOHR [19] and lacto-*N*-biose I (Galβ1 → 3GlcNAc) synthesised by a modification of the method of FLOWERS [8] were kindly supplied by Dr. A.S.R. DONALD, Clinical Research Centre, Harrow, U.K. Lacto-*N*-fucopentose III (Fucα1 → 3[Galβ1 →4]GlcNAcβ1 → 3Galβ1 → 4Glc) was a gift from Dr. V. GINSBURG, National Institutes of Health, Bethesda, Maryland, U.S.A.

Antisera

Anti-Le^a and anti-Le^b sera were raised in rabbits by immunisation with purified Le^a and Le^b active glycoproteins coupled with Shiga conjugated protein by a procedure essentially similar to that described for the production of immune rabbit anti-A sera [24]. Goat anti-Le^c and anti-Le^d reagents were prepared by immunisation with human saliva [11].

Glycosyltransferase assays

α-2-L-Fucosyltransferase assays were performed with phenyl-β-D-galactopyranoside as substrate as previously described [5]. For assays of α-L-fucosyltransferase 20 µl of the enzyme source (serum or saliva) was added to GDP-L-[^{14}C]fucose (0.2 nmol; 75,000 cpm) *N*-acetyllactosamine, 0.25 µmol, $MnCl_2$, 2 µmol, ATP, 0.5 µmol, and Tris-HCl buffer pH 7.2 5 µmol, in a total volume of 95 µl. α-4-L-Fucosyltransferase activity was assayed with the same reaction the same reaction mixture except that lacto-*N*-biose I was used as acceptor in place of *N*-acetyllactosamine. The mixtures were incubated for 16h at 37°C and the reaction products were chromatographed on Whatman DE81 paper developed for 48h with ethyl acetate-pyridine-

water (10:4:3 by vol.). The mobilities of the radioactive products, detected on a radiochromatogram scanner, were measured relative to standard oligosaccharides and the relevant areas of the chromatograms were then cut out and counted in a scintillation counter. The positional linkages of the transferred [^{14}C]-fucose residues were established by means of linkage specific α-2-**L**-fucosidase and α-3/4-**L**-α-3-*N*-acetyl-**D**-galactosaminyl- and α-3-**D**-galactosyl-transferase were assayed with 2'-fucosyllactose as the acceptor substrate by the methods described earlier [29].

Oligosaccharide inhibition tests

Haemagglutination inhibition tests with oligosaccharides of defined structure were carried out as described previously [34].

RESULTS

Family studies

Two unrelated families were referred for transferase assays because of blood group anomalies in one member of each family. The family pedigrees and ABO and Lewis red cell phenotypes of the family members are summarised in *Figs 1 and 2.*

The Hol. family (*Fig. 1*) were American blacks from Galveston,

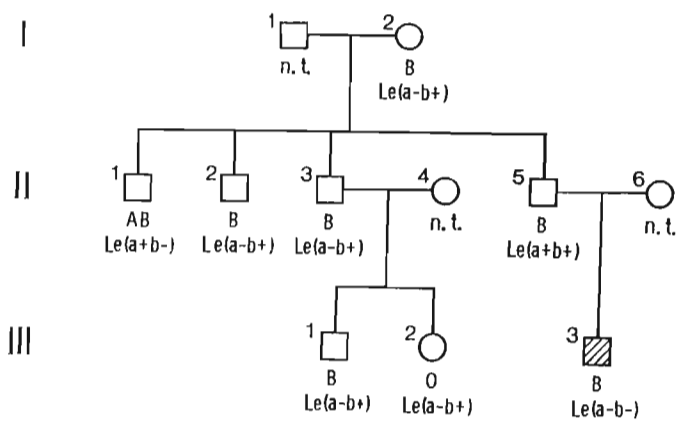


FIG. 1. — Hol. family. The hatched symbol indicates the family member lacking α-3-**L**-fucosyltransferase in serum and saliva.

Texas and studies were initiated because the Lewis red cell phenotype of II 5 was Le(a+ b+); a brief account has already appeared of the serological and glycosyltransferase results relating to this observation [6]).

The Mth family (*Fig. 2*) were South African blacks (Zulus) and interest in this family arose because II 4 appeared to be a dispermic chimaera with skin pigment mosaicism [7] and two populations of red cells; the serological and enzymic results pertaining to this red cell anomaly will be described elsewhere [23].

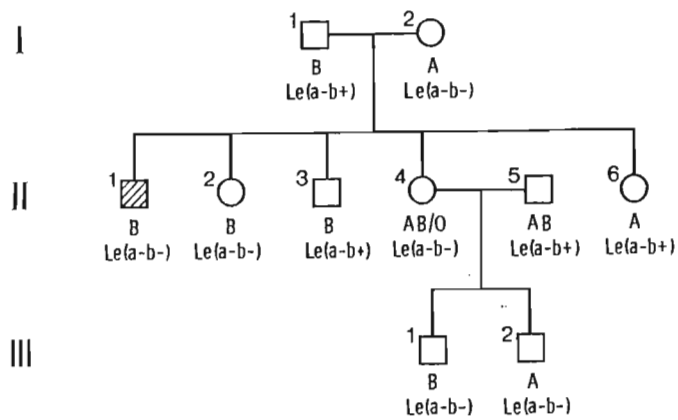


FIG. 2. — Mth family. The hatched symbol indicates the family member lacking α-3-L-fucosyltransferase in serum and saliva.

Glycosyltransferase studies

Saliva samples from the available members of each family were tested for α-3-L-fucosyltransferase activity with *N*-acetyllactosamine as acceptor and for α-4-L-fucosyltransferase activity with lacto-*N*-biose I as acceptor. Some samples were also tested for the α-3-activity measured with 2'-fucosyllactose as susbtrate which, like the α-4-activity detected with lacto-N-biose I, is dependent on the expression of the Lewis *Le* gene [17]. Although the levels of activity were in some instances very much lower than those found in control samples, all the salivas from the Lewis positive, Le(b+) or Le(a+), family members gave products with lacto-*N*-biose I and, where tested, 2'-fucosyllactose, whereas, as expected, these compounds failed to function as substrates for the enzymes in the salivas of the Lewis negative,

Le(a — b —), family members (*Table II*). However an unexpected finding was the lack of α-3-**L**-fucosyltransferase activity in two saliva samples, Hol III 3 and Mth II 1, when *N*-acetyllactosamine was used as the acceptor substrate (*Table II*). Increasing the enzyme incubation time from 16h to 64h failed to give any detectable product with

TABLE II

Fucosyltransferase activities in the salivas of Hol. and Mth. family members.

SALIVA SAMPLE		LEWIS GROUP	INCORPORATION OF [^{14}C]FUCOSE (pmol/h/ml)		
			α-3-**L**-Fucosyltransferase		α-4-**L**-Fucosyl-transferase Lacto-*N*-biose I acceptor
			N-acetyl-*lactosamine acceptor*	2'-Fucosyllactose acceptor	
Hol.	II 1	Le(a+b—)	34	30	67
	II 2	Le(a—b+)	24	12	15
	II 3	Le(a—b+)	7	4	5
	II 5	Le(a+b+)	34	10	25
	III 1	Le(a—b+)	3	4	4
	III 3	Le(a—b—)	0	0	0
Mth.	I 1	Le(a—b+)	5	n.t.	9
	I 2	Le(a—b—)	11	0	0
	II 1	Le(a—b—)	0	0	0
	II 2	Le(a—b—)	6	0	0
	II 3	Le(a—b+)	11	n.t.	18
	II 4	Le(a—b—)	6	0	0
	II 6	Le(a—b+)	7	2	2
Control salivas *					
1		Le(a+b—)	68	48	129
2		Le(a—b+)	72	29	162
3		Le(a—b—)	50	0	0

* From white U.K. donors.

this substrate. The demonstration of B (α-**D**-galactosyl)-transferase activity in the saliva of Hol III 3, comparable in level with that found in the saliva, collected and dispatched under the same condition, from his group B father, II 5 (*Fig. 1*), indicated that failure to find any fucosyltransferase activity did not result from the way the sample had been handled.

Saliva samples from Mth II 1 and his mother Mth I 2 were also tested for α-3-fucosyltransferase activity with lacto-*N*-neotetraose as acceptor. This substrate was utilised by an enzyme in Mth I 2's saliva but no activity was found in her sons saliva.

The H gene-associated α-2-**L**-fucosyltransferase is not expressed in detectable amounts in saliva [36] but examination of the sera of both Hol. III 3 and Mth II 1 showed that they each had levels of *H* transferase comparable with those found in the sera of other members of their families (*Table III*). The blood group A (α-*N*-acetyl-**D**-galacto-

TABLE III

Fucosyltransferase activities in the sera of Hol. and Mth. families.

SERUM SAMPLE		INCORPORATION OF [^{14}C]FUCOSE (pmol/h/ml)	
		α-2-**L**-Fucosyltransferase	α-3-**L**-Fucosyltransferase
		Phenyl β-**D**-galactoside acceptor	*N*-Acetyllactosamine acceptor
Hol.	II 1	60	45
	II 2	53	23
	II 3	120	90
	II 5	83	68
	III 1	90	38
	III 3	128	0
Mth.	I 1	56	n.t.
	I 2	71	20
	II 1	50	0
	II 2	45	22
	II 3	54	16
	II 4	35	10
	II 6	100	23
Control sera *			
OLe(a+b—)		86	80
OLe(a—b+)		70	32
OLe(a—b—)		93	91

* From white U.K. donors.

saminyl-) and B (α-**D**-galactosyl-)transferase activities in all the family members were those expected on the basis of their ABO red cell phenotypes. The sera of Hol III 3 and Mth II 1, however, were found to be completely deficient in α-3-**L**-fucosyltransferase activity (*Table*

III). Tests were performed on serum and saliva samples obtained from each of these individuals on two separate occasions separated by many months; no α-3-fucosyltransferase activity was found.

As a further check the buffy coat separated from the blood of Hol III 3 was assayed for α-2 and α-3-L-fucosyltransferase activities. No evidence of α-3-activity was found with *N*-acetyllactosamine as acceptor although α-2-activity was clearly demonstrable. A control buffy coat exhibited both α-2 and α-3-activities.

Survey of 2103 human sera for α-3-L-fucosyltransferase activity

In order to determine whether failure of expression of α-3-L-fucosyltransferase is more comon than previously supposed a survey was carried out on 2103 human serum samples. The two individuals who had been found to lack this enzyme activity were both from black families and both had the Lewis red cell phenotype Le(a — b —) (*Fig. 1 and 2*). The survey therefore included 842 samples from black donors; 676 samples were from South African Zulus donating at the Natal Blood Transfusion Service, Durban and 166 samples, generously supplied by Ms Phyllis Morel, American Red Cross Blood Services, Los Angeles, were from United States black donors. The Zulu donors were all grouped for ABO and the sex of the donors was recorded. In addition, 132 of the 356 group Os were Lewis typed and shown to include 23 Le(a— b—), 26 Le(a+ b—) and 83 Le(a— b+) individuals. The U.S. black donors comprised 100 group Os, 13 group As and 53 ABs. The remaining samples were from randomly selected white donors of South African (61) and United Kingdom (1200) origin, all of known ABO groups. Although the range of levels of α-3-L-fucosyltransferase activity was very wide, no other examples of sera completely deficient in this enzyme activity were disclosed by this survey.

All the sera were tested for both α-2 and α-3-L-fucosyltransferase activities. The sample from South Africa were freshly taken and dispatched by air to the Clinical Research Centre under carefully controlled conditions; a comparison of the levels of activity in the samples from the two ethnic groups is therefore valid. The tendency towards higher levels of α-2-L-fucosyltransferase activity in groups A and AB sera than in groups B or O, which had been observed earlier [5, 25], was confirmed for both the black and the white donors (*Table IV*). No such correlation was observed between α-3-L-fucosyltransferase levels and the ABO groups of the donors (*Table IV*) and the mean levels of this activity were similar in males and females. The black population had more samples at the lower end of the

range of α-3-L-fucosyltransferase activity than the white population (*Table IV*). Only one of the sera from the 61 white South African donors incorporated less than 30 pmol [^{14}C]fucose into *N*-acetyllactosamine per hour per ml of serum whereas 11% of the 676 Zulu samples incorporated less than 30 pmol/h/ml and some sera gave values as low as 2 pmol/h/ml (*Table IV*). The low values for α-3-L-fucosyltransferase among the Zulu sera could not be correlated with the Le(a — b —) phenotype where this was known.

TABLE IV

Fucosyltransferase levels in serum samples from South African populations.

POPULATION	ABO BLOOD GROUP	NUMBER TESTED	α-3-L-FUCOSYL-TRANSFERASE (pmol/h/ml)		α-2-L-FUCOSYL-TRANSFERASE (pmol/h/ml)	
			Mean	Range	Mean	Range
Black (Zulu)	O	356	72	4-224	48	16-176
	A	174	64	5-144	128	32-256
	AB	21	72	2-176	112	16-205
	B	125	64	16-176	96	16-160
White	O	31	72	32-191	96	32-224
	A	27	80	32-192	144	48-240
	B	3	48	16-80	112	96-128

The general picture obtained for the distribution of α-2- and α-3-L-fucosyltransferases in the samples from the U.S. black donors were similar in that the α-2-enzyme varied according to the ABO groups of the donors whereas the α-3-L-fucosyltransferase did not. Seventy-eight per cent of the sera incorporated less than 30 pmol/h/ml of [^{14}C]fucose into the 0-3 position of the *N*-acetylglucosamine residue in *N*-acetyllactosamine; however, although carefully dispatched in the frozen state these samples had been stored previously and may have lost some activity on storage. None of the 1200 U.K. whites tested had α-3-L-fucosyltransferase activities of less than 30 pmol [^{14}C]fucose/h/ml.

ABH secretor status and Lewis Le^c and Le^d red cell phenotypes of Hol III 3 and Mth II 1

Both Hol III 3 and Mth II 1 were grouped as Le(a — b —) on the basis of red cell tests. Saliva inhibition tests showed that Hol III 3

was a non-secretor whereas Mth II 1 was a secretor of B and H. Neither saliva had Le^a or Le^b activity.

A sample of Hol III 3's blood was tested at Ortho Diagnostics, Raritan, U.S.A. with goat ant-Le^a, Le^b, Le^c and Le^d reagents [11]; the cells grouped as Le(a — b — c — d —) [6]. Mth II 1's red cells, tested at the Clinical Research Centre, Harrow, U.K. with rabbit ante-Le^a and Le^b reagents and goat anti-Le^c and Le^d sera also grouped as Le(a — b— c— d—). Saliva from Hol III 3 and Mth II 1 failed to inhibit rabbit anti-Le^a or Le^b reagents or the goat anti-Le^c or Le^d sera.

Oligosaccharide inhibition of goat anti-Le^c serum

The agglutination of the red cells of an Le(a — b —) non-secretor donor by the goat anti-Le^c serum was tested for inhibition with a range of low-molecular-weight oligosaccharides of defined structure (*Table V*). Weak inhibition was observed with 3-fucosyllactose and definite inhibition with lacto-*N*-fucopentaose III. Closely related oligosaccharides lacking α-3-linked fucose failed to show inhibition.

TABLE V

Oligosaccharide inhibition tests with goat anti-Le^c serum.

OLIGOSACCHARIDE		MINIMUM AMOUNT GIVING COMPLETE INHIBITION OF AGGLUTINATION * (μg)
NAME	STRUCTURE	
Lacto-*N*-tetraose	Galβ1 → 3GlcNAc	200
Lacto-*N*-biose I	Galβ1 → 3GlcNAcβ1 → 3Galβ1 → 4Glc	400
Lacto-*N*-neotetraose	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc	400
2'-Fucosyllactose	Fucα1 → 2Galβ1 → 4Glc	> 400
3-Fucosyllactose	Fucα1 → 3[Galβ1 → 4]Glc	100
Lacto-*N*-fucopentaose III	Fucα1 → 3[Galβ1 → 4]GlcNAcβ1 → 3Galβ1 → 4Glc	3

* Haemagglutination of group O Le(a— b—) non-secretor red cells by goat anti-Le^c serum.

DISCUSSION

Two unrelated individuals have been found who lack in both their sera and saliva the enzyme that catalyses the transfer of L-fucose to the *O*-3 position of *N*-acetylglucosamine. The expression of this enzyme is not dependent on the Lewis gene [17] and it had previously

been thought to be present in everyone since no individual lacking this fucosyltransferase in serum had been seen on examination of many samples over a period of more than ten years. The two individuals in whom the enzyme was missing were black, one from South Africa and one from the U.S.A. A systematic survey was therefore undertaken of more than 2000 serum samples including 842 from South African and American black donors. Both deficient subjects also belonged to the Le(a — b —) phenotype and, although the expression of the α-3-L-fucosyltransferase in other members of the Mth family with this phenotype excluded a direct relationship between the two observations, care was taken to include known Le(a — b —) donors in the sample. The results revealed a wide range in the level of activity of the enzyme in the sera from both black and white donors, with some very low levels among the sera from the black donors. However, no other examples were found of sera completely deficient in α-3-L-fucosyltransferase activity. Failure to express this enzyme is therefore very rare in both black and white populations.

Serological examination of the red cells and saliva of the two individuals deficient in α-3-L-fucosyltransferase revealed a second very rare condition in that their red cells grouped as Le(a — b — c — d —) and Le^a, Le^b, Le^c and Le^d activities were also missing from their saliva. No one with this phenotype had been reported previously. Hence the only two known individuals lacking detectable α-3-L-fucosyltransferase activity also lacked Le^c and Le^d antigenic determinants. A relationship between these two rare anomalies was suspected in view of the earlier indications that α-3-linked fucose is involved in the specific determinant recognised by anti-Le^c reagents [14, 9]. These proposals have now been strengthened by the isolation from Le(a — b —) plasma of the Le^c-active glycolipids containing the 3-fucosyllactosamine structure [16]. Quantitative comparisons between the haemagglutination inhibition given by glycolipids and oligosaccharides are difficult to make, especially when tests are performed in different laboratories by slightly different procedures. In the inhibition tests carried out in the course of this investigation with goat anti-Le^c and low-molecular-weight oligosaccharides (*Table V*), the compounds with unsubstituted Type 1 structures (lacto-*N*-biose I and lacto-*N*-tetraose) failed to show any inhibition whereas definite binding occurred with the compounds containing Type 2 structures substituted on the subterminal sugar with α-3-linked fucose residues (3-fucosyllactose and lacto-*N*-fucopentaose III). The greater activity found by HANFLAND *et al.* [16] for the glycolipid (GL-6) bearing a branched oligosaccharide comprising an unsubstituted Type 1 chain ending and a fucosyl-substituted Type 2 chain than for the simpler

Type 2 glycolipid GL-3 indicates a role for the Type 1 grouping in ensuring enhanced binding to the Le^c antibody, but the present evidence strongly supports the inference that an α-3-linked **L**-fucosyl-residue is an essential part of the Le^c determinant.

The discrepancies reported concerning the specificity of reagents detecting antigens present on the red cells and in saliva of individuals grouped as Le(a— b—) non-secretors can, in part, find an explanation in the types of non-reducing structures that are likely to terminate the oligosaccharide chains of the glycoconjugates synthesised by persons with this phenotype (*Table VI*). In epithelial tissues of Le(a— b—) non-secretors neither the Lewis *Le* gene-associated α-3/4-

TABLE VI

Possible terminal non-reducing structures in oligosaccharide chains of glycoconjugates of individuals with the Le(a— b—) non-secretor phenotype.

Type 1 chain	Galβ1 → 3GlcNAc...
	NeuAcα2 → 3/6Galβ1 → 3GlcNAc...
Type 2 chain	Galβ1 → 4GlcNAc... ↑ 1,3 Fucα
	NeuAcα2 → 3/6Galβ1 → 4GlcNAc...
	NeuAcα2 → 3/6Galβ1 → 4GlcNAc... ↑ 1,3 Fucα

L-fucosyltransferase nor the *H*(*Se*) gene-associated α-2-**L**-fucosyltransferase are expressed. Thus both the Type 1 (Galβ1 → 3GlcNAc) and Type 2 (Galβ1 → 4GlcNAc) chain endings lack the Fucα1 → 2Gal terminal structures that confer H specificity and the Type 1 chains lack the Fucα1 → 4[Galβ1 → 3]GlcNAc and Fucα1 → 2Galβ1 → 3-[Fucα1 → 4]GlcNAc structures that confer, respectively, Le^a and Le^b specificities. The type 1 chain ending could remain unsubstituted and thus be available as an antigenic structure not normally exposed on the glycoconjugates of those expressing an *Le* gene. Alternatively, if a sialyltransferase is present in the cell, the terminal β-**D**-galactosyl residue of the Type 1 chain might become an acceptor for this enzyme (*Table VI*) and another antigenic structure could thereby be synthesised that is not normally expressed when the secretor *Se* and *H* genes are functional. In the majority of Le(a— b—) individuals, where the α-3-**L**-fucosyltransferase is active, the Type 2 chain endings are available for substitution with **L**-fucose on the *O*-3 position of *N*-acetyl-glucosamine (*Table VI*) to give the 3-fucosyllactosamine structure. In secretors expressing the α-2-**L**-fucosyltransferase a difucosyl structure

would normally be synthesised (Fucα1 → 2Galβ1-4[Fucα1 → 3]GlcNAc) in which the 3-fucosyllactosamine structure is masked. The unsubstituted terminal β-**D**-galactosyl unit could again be an acceptor for a sialyltransferase to give either sialyllactosamine or a hybrid sialylfucosyl structure (*Table VI*). Thus a number of structures might appear to arise *de novo*, or be present at a much higher density, in the glycoconjugates from Le(a— b—) non-secretors than in those from Le(a— b—) secretors or from Le(a+b—) or Le(a— b+) individuals. An antibody raised against any one of these structures, either as part of the glycoconjugates present in the secretions from an Le(a— b—) non-secretor, or as an artificial antigen made from a defined oligosaccharide hapten, might react preferentially with the red cells of an Le(a— b—) non-secretor and thus be termed an "anti-Le^c" reagent.

The involvement of the 3-fucosyllactosamine structure in the Le^c determinant raises further problems concerning the confused nomenclature relatig to the Lewis groups in general and the 3-fucosyllactosamine structure in particular. First isolated from ovarian cyst glycoprotein [22] but not given a name, this same structure was subsequently found in a glycolipid and termed X to distinguish it from the structurally related Le^a determinant [15]. This symbol has continued to be used although the preface "Le" is now frequently added. The description Le^x to denote the 3-fucosyllactosamine structure is clearly incorrect as this term was pre-empted many years ago [2] for an antibody detecting a structure common to Le^a and Le^b determinants [31]. 3-Fucosyllactosamine was found to be the determinant recognised by a monoclonal antibody SSEA 1 [10] and the term SSEA 1 is now sometimes used also designate this structure wherever it occurs, although as SSEA stands for "stage specific embryonic antigen" this designation is scarcely appropriate. Clearly there is a need for rationalisation of the nomenclature of the 3-fucosyllactosamine structure and for a clarification of the relationship of this structure to the Lewis blood group system.

SUMMARY

1. Two unrelated individuals are reported who lack α-3-**L**-fucosyltransferase activity in their serum and saliva. Both were blacks, one from the United States and the other from South Africa. No other of the tested members of their families lacked this enzyme.

2. A survey of more than 2000 serum samples from both black and white South African blood donors, black United States donors and white United Kingdom donors failed to disclose another example of a serum deficient in α-3-**L**-fucosyltransferase activity.

3. The two individuals lacking in α-3-**L**-fucosyltransferase activity both had the Lewis blood group phenotype Le(a— b— c— d—). No other persons with this phenotype have been reported.

4. The absence of Le^c activity in the two individuals who are deficient in α-3-**L**-fucosyltransferase is consistent with the interpretation that α-3-linked **L**-fucose is an essential part of the antigenic determinant recognised by the anti-Le^c reagent used in this investigation.

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Dr. W.M. WATKINS,
Division of Immunochemical Genetics,
MRC Clinical Research Center,
Watford Road, Harrow,
Middlesex HA 1 3UJ,
U.K.

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Paper 24

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The Secretion of A, B, H and Lewis Blood Group Substances in the Gastric Juice and Saliva of Chacma Baboons (*Papio ursinus,* Kerr) and Vervet Monkeys (*Cercopithecus pygerythrus,* Cuvier)[1]

H.J. Downing, P.P. Moores, M.C. Bolstridge, M.E. Schleyer, H.J. Klomfass and G.R. Davidson

Natal Institute of Immunology, Durban

Abstract. Results of tests for the presence of blood group substances A, B, H, Le^a and Le^b in saliva and gastric juices of chacma baboons and in vervet monkeys are reported.

Key Words
Chacma baboons
Vervet monkeys
Blood groups
A, B, H blood groups
Lewis blood groups

Le and A, B, H Substances in Primates

Five blood group-specific substances, A, B, H, Le^a and Le^b are detectable in human secretions by means of agglutination inhibition tests with the appropriate erythrocytes and antibodies. The saliva of chacma baboons *(Papio ursinus,* Kerr) has been studied for the presence of A, B and H substances [7, 20] but not for Lewis substance although this specificity has been detected in other species of baboon [20]. A, B, H and Lewis specificities have been studied in the saliva of vervet monkeys (*Cercopithecus pygerythrus,* Cuvier) [20] but in neither baboons nor vervet monkeys has there been a study of these specificities in gastric juice.

We report here an investigation of A, B, H, Le^a and Le^b substances in the saliva and gastric juice of 21 chacma baboons and 23 vervet monkeys from Natal and Transvaal.

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Materials and Methods

1. Species

The taxon *Papio ursinus*, Kerr, is regarded [11] as being a distinct species belonging to the superspecies *P. cynocephalus*, Linn. This species is not restricted to one ecosystem but inhabits a number of vegetational and climatic zones including the areas from which the baboons used in this investigation were captured. The classification of the baboons studied was confirmed by means of the relevant taxonomic characteristics such as the callosities on the posterior end [11].

The vervet monkey (*Cercopithecus pygerythrus*, Cuvier) is a species of the superspecies *C. aethiops* of which the superspecific type species is the Grivet monkey. HILL [10] recognizes thirteen subspecies of *C. pygerythrus*. No attempt has been made in this paper to classify beyond species although the geographical distribution suggests that the specimens concerned were of the subspecies *C.p. pygerythrus*.

2. Anaesthetics

The animals were anaesthetised by an intramuscular injection of phenylcyclidine hydrochloride ('Sernylan' Parke, Davis & Co.), 1 mg/kg body weight. Where necessary, Diazepam ('Valium', Roche Products Ltd.) was administered intravenously (2.5 mg/kg) to counteract muscle spasm.

3. Collection of Saliva Samples

Salivation was induced by an intramuscular injection of pilocarpine nitrate (Boehringer), 0.2 mg in aqueous suspension per kg body weight. Atropine was used if excessive bronchial secretion occurred. The animals were placed face downwards and the saliva collected in plastic bags, transferred to test tubes which were kept in a boiling water bath for 20 min to prevent enzymatic hydrolysis of the glycoproteins. The samples were then centrifuged and the supernates stored at -20°C.

4. Collection of Gastric Juice

Gastric juice was collected from fasting baboons by means of a Ryle tube inserted through the mouth. No chemical stimulants were used but gentle pressure was applied to the abdomen. In the vervet monkeys it was necessary to induce gastric secretion by an intravenous injection of Pentagastrin ('Pentavlon' ICI, Cheshire), dose 6 mg/kg body weight. The samples were boiled, dialysed against phosphate-buffered saline pH 7.2, centrifuged, and the supernates stored at -20°C.

5. Inhibition Test for A, B, H Substances

The titre of A, B, H blood group substances in the saliva and gastric juice was assessed by the doubling dilution agglutination-inhibition test. [4] The anti-A reagent had a titre of 128 when tested with A_2 cells, and the anti-B had a titre of 256. Both were diluted 1 in 10 with saline before use. The anti-H was an extract of *Ulex europaeus* seeds diluted 1 in 6 with saline.

6. Inhibition Test for Lewis Substances

The anti-Le^a and anti-Le^b sera had titres of 16 and 8, respectively, by the ficin test. The anti-Le^a serum was obtained from a Negro female donor of blood group O rh Le

(a–b–). The Le^b serum used was from a Negro male donor of blood group A_1 Rh_0 Le(a–b–). The specificity of both antisera was confirmed by testing them in parallel with three known anti-Le^a and eight known anti-Le^b reagents on random blood samples from 98 Negroes and 55 Caucasians. No discrepant results were obtained. The Negro samples tested consisted of 24 Le(a+b–), 52 Le(a–b+) and 22 Le(a–b–), and the Caucasian samples of 16 Le(a+b–), 36 Le(a–b+) and 3 Le(a–b–), which is in keeping with the expected frequencies for random samples from these two races. The serum was not inhibited by salivas containing H substance but not Le^a or Le^b substances from persons of group Le(a–b–).

In the test, one drop of saliva and one drop of antiserum were mixed on a glass tile and allowed to stand at approximately 22 °C in a moist chamber for 30 min. One drop a 0.25 percent preparation of ficin in buffered saline and one drop of a 5- to 8-percent suspension of the appropriate red cells in saline were then added. After a further 60 min in the moist chamber, readings were made macroscopically by gently tilting the tile over a source of light.

7. Statistical Analyses

The results were analysed by parametric and non-parametric statistics. In the parametric statistics, the mean and standard deviation were calculated for each set of data, and the baboon results were then compared with those for the vervet monkeys by testing the significance of the difference between the means. The same method was used to compare the titres of each type of blood group substance in saliva and gastric juice within each species. A correlation coefficient was also calculated for the data.

In the non-parametric statistics, it was assumed that the presence or absence of blood group substances in the various secretions was genetically determined and that the baboons and vervet monkeys could be classified as secretors or non-secretors accordingly. Each blood group specificity was investigated in turn, and the results for saliva and gastric juice were tabulated in a 2×2 contingency table to which Fisher's Exact Test was applied.

Results

The titres obtained from the various inhibition tests on the secretions of the baboons and vervet monkeys are listed in table I. The contingency tables and probabilities based on Fisher's Exact Test for comparing the presence of blood group substances in saliva and gastric juice are shown in table II. The means, standard deviations, correlation coefficients and t tests for the various blood group substances in saliva and gastric juice of both species are tabulated in table III.

The results in table I show that there were no samples of saliva or gastric juice in either of the species that had a titre of 1 in the inhibition test. All the samples either failed to inhibit agglutination when undiluted, or were able to inhibit at dilutions of at least 1 in 2.

Table I. Results of inhibition tests for A, B, H and Lewis substances in saliva and gastric juice of baboons and vervet monkeys

Substances secreted	Numbers of baboons and vervet monkeys with similar titre end-points												
	0	1	2	4	8	16	32	64	128	256	512	1,024	2,048
In baboon saliva													
A	3								1		2		15
B	10							1	2	3	3	1	1
H				1				3	12	3	2		
Le[a]	21												
Le[b]	8		5	3		2	3						
In baboon gastric juice													
A	1			2	3		2	2	2	2	1	1	5
B	8		2	1	2		1			2	3	2	
H	4			4	4	2	2	2	2		1		
Le[a]	16		1	1		3							
Le[b]	13		3	2	1		1			1			
In vervet monkey saliva													
A	2		1		3		5	2	3	4	2	1	
B	16		3			1	2		1				
H						1	4	3	9	4	2		
Le[a]	2		1		1	4	5	7	1		1		1
Le[b]	1								4	5	6	3	4
In vervet monkey gastric juice													
A			1	1	2	2	3	7	3	2	1	1	
B	14		2				2		2	1		1	1
H	8		5		4	3	2	1					
Le[a]	17		1	1			2	1	1				
Le[a]	15				2				3	2			1

It was on the basis of this finding that contingency tables shown in table II were prepared. Nearly all the samples of saliva and gastric juice of both species contained A substance. Of the 21 baboons examined, only three did not show A specificity in their saliva; however, two of these did show A specificity in their gastric juice. Thus, only one baboon out of 21 failed to show A specificity in either secretion. All of the 23 vervet monkeys secreted A substance in their gastric juice, but two lacked this specificity in their saliva.

B substance occurred less frequently. Seven baboons and 14 vervet monkeys did not have B substance in either of their secretions, while

Table II. Contingency tables and probabilities based on Fisher's Exact Test for comparing the presence of blood group substances in saliva and gastric juice

Substances secreted	Saliva/gastric juice ++	+−	−+	−−		Probability
In baboons						
A	18	0	2	1	(1)	<0.05
B	10	1	3	7	(2)	<0.05
H	17	4	0	0	(3)	<0.05
Le^a	0	0	5	16	(4)	<0.05
Le^b	7	6	1	7	(5)	<0.05
Le^a or Le^b	7	6	3	5	(6)	<0.05
In monkeys						
A	21	0	2	0	(7)	<0.05
B	7	0	2	14	(8)	<0.05
H	15	8	0	0	(9)	<0.05
Le^a	6	15	0	2	(10)	<0.05
Le^b	8	15	0	0	(11)	<0.05
Le^a or Le^b	8	14	0	1	(12)	<0.05

10 baboons and seven vervet monkeys possessed this specificity in both secretions. H substance was present in all the samples of saliva of both species but it was not present in the gastric juice of four baboons and eight vervet monkeys. Le^a specificity was not found in the saliva of any of the baboons and was present in the gastric juice of only five of them. By contrast, Le^a specificity was found in the saliva of 21 vervet monkeys, but only six of these possessed Le^a substance in their gastric juice. Although all the vervet monkeys secreted Le^b substance in their saliva, only eight secreted it in their gastric juice. Thirteen baboons secreted Le^b substance in their saliva and seven of these secreted it in their gastric juice also. Seven baboons did not possess this specificity in either secretion.

Of the five specificities tested, A, B, H, Le^a and Le^b, the presence of B substance in the saliva of both species was significantly related to its presence in the gastric juice of that species. There was no similar relationship for A, H, Le^a or Le^b substances.

Quantitative analysis of the five specificities showed that the titres of B substance in the saliva of baboons and vervet monkeys correlated with the titre in the gastric juice of the species. A similar relation-

Table III. The means, standard deviations, correlation ceofficients and t tests for A, B, H,Le^a and Le^b substances in the saliva and gastric juice of baboons and vervet monkeys

Specificity	Baboon			Vervet monkey			t test baboon/vervet	
	saliva	gastric juice	correlation coefficient	saliva	gastric juice	correlation coefficient	saliva	gastric juice
A	1.518 ± 848	608 ± 837	0.223	164 ± 236	132 ± 221	0.033	7.071[1]	2.527[1]
B	271 ± 473	198 ± 323	0.705[1]	9.3 ± 27.0	159 ± 455	0.608[1]	2.534[1]	0.330[1]
H	253 ± 288	50 ± 100	0.311	154 ± 133	9.5 ± 14.9	0.102	1.441	1.673
Le^a	0	3 ± 5.6		160 ± 435	11.4 ± 29.2	0.121		1.324
Le^b	7 ± 11	15 ± 54	0.489[1]	733 ± 676	128.7 ± 416.7	0.332	5.037	1.268

[1] Significant at 5% level.

ship could be shown for the titre of Le^b substance in the secretions of the baboons. As in every case, there was a large number of baboons and vervet monkeys without Le^b substance in either secretion, the difference may be qualitative rather than quantitative. When the correlation coefficients were recalculated excluding the double negatives, the results were no longer significant. The titres of A and B substances in the saliva of the baboons and of A substance in gastric juice were significantly higher than in the vervet monkeys. For Le^b substance the situation was the reverse; the titres in the saliva of the vervet monkeys were significantly higher than in the saliva of the baboons.

Discussion

The presence of H substance in the saliva of all the baboons and vervet monkeys is consistent with the findings of other authors for these species [21]. The frequencies of A and B specificities in the saliva of the populations from which the present animals were selected have been reported elsewhere [6, 7]. We were aware that caution should be exercised in using sera categorized in one species to classify antigens in another species [1, 2] and that this may be especially true of the Lewis antigens where a serum that appears to be specific anti-Le^b may actually contain anti-H antibodies of the type not readily inhibited by soluble H substance [22]. With these reservations in mind, the results of our experiments were considered in terms of biosynthesis of the various blood group glycoproteins.

The biosynthetic pathways of A, B, H and Lewis substances have been described by WATKINS [18]. According to her hypothesis, one polysaccharide is converted to another by the addition of the appropriate monosaccharide, and each reaction requires the presence of a specific glycosyltransferase provided by a blood group gene. The formation of H substance is the result of the appropriate fucosyltransferase, produced by the *H* gene [15], acting upon a precursor glycoprotein. Similarly, the fucosyltransferase produced by the *Le* gene [8] converts this same precursor glycoprotein to Le^a substance. Both of the fucosyltransferases, acting further upon the precursor, convert it to Le^b substance which is thus produced by the combined action of the two genes, *H* and *Le*. H substance is the immediate precursor of both A and B substances to which it is converted by N-acetylga-

lactosaminyltransferase [9, 13, 16] and α-galactosyltransferase [12], respectively.

In human beings, the expression of the *H* gene in secretory tissue is influenced by an *Se* gene inherited independently. Persons homozygous for its recessive counterpart *se* (about 20% of the population), do not synthesize the fucosyltransferase which in turn synthesizes H substance from the precursor. In nonhuman primates, the expression of the *H* gene appears to be independent of the secretor gene as H substance is uniformly present in the saliva. The absence of H substance from the gastric juice of four baboons and eight monkeys poses the question whether or not *se* operates in the gastric mucosa of this species, but as each of the animals concerned secreted A and/or B substance in its gastric juice which would require the synthesis of H substance as a precursor, it apparently does not. Another possibility is that insufficient H substance remained unconverted to A and/or B substances to be detected. The failure to detect Le^a substance in the saliva of five baboons possessing this specificity in their gastric juice may be attributed similarly to conversion of all the available Le^a to Le^b substance, which was present in each case.

It is more difficult to explain the presence of Lewis substances in the gastric juice of baboons which did not secrete them in their saliva. A similar observation was made by WARD [17] who found both Le^a and Le^b substance in the gastric juice of some baboons which did not secrete either specificity in their saliva. WARD's findings differed from ours in that she detected Lewis substances in the gastric juice of all the baboons tested, whereas we found them in only 40%. WARD, however, extracted the gastric secretions from the stomach mucosa of her baboons at autopsy, whereas we obtained our samples from the live animals. The difference may be due in our case to dilution of the substances in the gastric juice. It could also explain our failure to detect Lewis substances in the gastric juice of six baboons and 14 monkeys that possessed them in their saliva.

No correlation was found between the amount of each of the substances A, B, H in saliva and in gastric juice although such a relationship has been reported for human secretions [3, 14]. A correlation has also been reported between the amounts of blood group substances in human saliva and in milk [5]. DENBOROUGH [3], however, expresses the amount of blood group substances as a 'secretor-score' whereas we have used the actual titre. MCCONNELL's [14] results are expressed

as titres, but WIENER [19] has challenged the validity of his calculations.

Possibly, biochemical rather than serological methods are required for a conclusive quantitative analysis. The results of the present serological investigation are nevertheless in keeping with current theories on the biosynthesis of the A, B, H, Le^a and Le^b substances in the secretions of primates.

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Request reprints from: Dr. H.J. DOWNING, Natal Institute of Immunology, 149 Prince Street, *Durban, Natal* (South Africa)

CHAPTER VII

ANTIGENS NOT YET ASSIGNED TO SYSTEMS

VII.1 PAPERS

The new McCoy antigen McC^e: further expansion of the York, Cost, McCoy, Knops blood group system
Paper 38 by Molthan, Moores and Hartwell

Wd(a+) red blood cells in two sisters of a Hei//om Khoisan family in Namibia
Paper 48 by Moores, Smart, Marks and Botha

Congenital dyserythropoietic anaemia (Type II) presenting with haemosiderosis
Paper 42 by Bird, Jacobs and Moores

VII.2 INTRODUCTION

The McCoy antigens are included in the collection which contains those whose antibodies to some extent have questionable clinical significance [Issitt, 1985]. The collection also includes Ch^a, Rg^a, Bg^a, Bg^b, Bg^c, Sd^a, Wr^a, Cs^a, Yk^a, Kn^a, JMH and some other antigens. The first antigen in the McCoy series, McC^a, was described in 1978 by Molthan and Moulds. Moulds, Moulds and Molthan [1979] and Molthan [1983] have since added McC^b, McC^c, McC^d, McC^e and McC^f. All six McCoy antigens have different frequencies in both Blacks and Whites. As none of the corresponding antibodies agglutinate rare McCoy/Knops null red cells, these cells are useful for their preliminary identification.

The low frequency antigen, Wd^a, was first reported from Canada in 1981 by Lewis and Kaita. It was named after the Waldner family of Schmiedeleut Hutterians. The Hutterians, who originated as a group in central Europe and later moved to Russia, had settled in Canada in the latter part of the 19th century. Wd(a+) red cells were also identified but not recorded in a Dutch family in Holland.

Wiener, Unger, Cohen and Feldman [1956] were the first to describe the antigen I and its corresponding antibody, anti-I.

The red cells of most adults were I+ and those of a few I-. In 1960, Marsh and Jenkins, and in 1961, Marsh, identified anti-i. The red cells of the newborn, those of young children up to 18 months of age and those of I- adults had large amounts of i antigen. Both I and i were demonstrable to some extent on most red cells; their levels changed reciprocally as the red cells matured and in response to diseases such as anaemia.

VII.3 COMMENTARY

VII.3.1 Attainments in Durban, 1961 to 1991

VII.3.1.1 The McCoy antigen McC^e

In 1973, June Humphreys of Vancouver, Canada, kindly sent the author a sample of serum containing two antibodies: anti-Targett and anti-Fy^a. To the author's surprise, the serum agglutinated the red cells of large numbers of Fy(a-b-) Natal Blacks. At the time, Professor M. Lewis (Rh Laboratory, Winnipeg, Canada) happened to be preparing a paper about the Tar+ phenotype. Believing that the Natal Blacks had Tar+ red cells, she made some pertinent absorption-elution studies with the serum and identified a third antibody. On hearing this, the author nick-named this antibody "anti-Zulu". Some years later, Dr L. Molthan (Miller Memorial Blood Centre, Bethlehem, USA) inquired of the author whether she might publish her own results with the "Zulu" antibody. As she had prepared some fine family and antigen frequency studies, it seemed best to combine everything in one paper and this was agreed. Paper 38 described 17 further examples of "anti-Zulu", none of which contained anti-Tar. As Dr Molthan had identified the corresponding antigen as another in the McCoy series, in place of "Zulu", the author suggested the name McC^e. The antibody, anti-McC^e, was type IgG and reacted only by the indirect antiglobulin technique. It agglutinated red cells from +^{w} to 2+, the stronger results generally occurring in the Blacks. As both Blacks and Whites had McC(e+) red cells, the White patients who made anti-McC^e need not necessarily have received a blood transfusion from a Black donor. All McC(e+) red cells were McC(a+), but the majority of McC(a+) red cells were McC(e-). The genetics of the McCoy antigens still has to be

resolved.

VII.3.1.2 The Waldner antigen Wd^a

Rare Wd(a+) red cells were surprisingly identified in two sisters of a Hei//om family in northern Namibia [paper 48]. The Hei//om are a Khoisan people whose genetic markers show that they are Khoi rather than San in origin. At the time, the authors were studying a group of 114 Hei//om as part of their ongoing search for the source of the Dantu gene complex. Among the reagents used was a control serum known to contain anti-S and at least nine antibodies for low frequency antigens, among which was anti-Wd^a. The gene encoding *Wd^a* might have arisen independently in the Hei//om. Alternatively, it might have been acquired by them through miscegenation with Europeans. Prior to the 1914-1918 war, miscegenation between German men and Khoisan women in South West Africa had been common. As the Hei//om were nomadic hunter-gatherers, their ancestors were unlikely to have acquired the *Wd^a* gene from the early White farming community at the Cape of Good Hope.

Four Hei//om were also found to have Rh:-34 red cells. This phenotype had been associated thus far primarily with the Black race.

VII.3.1.3 I and i antigens

In congenital dyserythropoietic anaemia (CDA), type II, the amount of I antigen on the red cells is decreased and the amount of i antigen markedly increased. In the first case to be reported from South Africa [paper 42], the authors found, as expected, that the Ham's acid lysis test was positive and sucrose lysis test negative. Titrations were made with suitable anti-I and anti-i reagents. The proposita's red cells scored 15 with anti-I (normal score with adult red cells 32-58 and with cord cells 0-2), and scored 60 with anti-i (normal score with cord cells 57-68 and with adult red cells 0-7). Both anti-I and anti-i also haemolysed her red cells in the presence of complement. In the family study, besides the

proposita, five members were found to have unusual expression of I and i. The inheritance was explained by suggesting that the proposita had a double dose of a recessive gene which encoded CDA, type II. In the heterozygous state, the gene partially depressed the expression of I and partially enhanced the expression of i antigen.

Paper 38

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Subject interest
BLOOD GROUP SEROLOGY

The new McCoy antigen McC^e: further expansion of the York, Cost, McCoy, Knops blood group system

LYNDALL MOLTHAN,*‡ PHYLLIS MOORES† AND GEORGIANNE HARTWELL

**Miller Memorial Blood Center, 2100 Westgate Drive, P.O. Box 2867, Bethlehem, Pennsylvania 18001, USA., †Natal Institute of Immunology, P.O. Box 2356, Durban 4000, South Africa, and Central Blood Bank of Pittsburgh, 812 5th Avenue, Pittsburgh, Pennsylvania 15219, USA.*

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McC^e, the fifth McCoy specificity of human red blood cells, is presented. The corresponding antibody, anti-McC^e, was encountered in the sera of 17 previously transfused Whites. The original example was identified as a second antibody in the original anti-Tar, when this serum was seen unexpectedly to agglutinate the cells of many Negroes: this second antibody had been nicknamed 'anti-Zulu' in 1973. Its true specificity as anti-McC^e has evolved since 1979 through the study of random and selected populations.

The frequency of McC^e is 0.3504 in 371 American Negroes and 0.0983 in 722 American Whites. Not one of 39 consecutive unrelated McCoy/Knops null individuals tested was McC(e +) nor were 52 McC(a −) and Kn(a +) persons, but all 29 random McC(a + d −) Negroes and 36 of 60 McC(a + d −) Whites tested were McC(e +). Informative pedigrees confirm that the new antigen is another in the McCoy series.

Key words: Blood groups. Isoantibodies. Isoantigens

Introduction

This report presents accumulated data on the study of a new antigen McC^e, based on 17 examples of anti-McC^e and corresponding antigen determinations in 722 random American White blood donors, 371 random American Negro donors and five informative pedigrees. This study encompassed the period 1979 through 1981.

In the spring of 1979 blood samples from a White patient, JG, were referred for identification of an allo-antibody which reacted preferentially with red cell samples from Negroes. The serum reactions did not fit for V, VS, Js^a nor the Bg antigens and initially the reactivity rate was 25% of a small series of Negro bloods. In July 1979 the original anti-Tar serum, Dob,[1,2] was distributed by the Vancouver BC Red Cross through the SCARF program.[3] It was stated that this serum also contained an antibody directed against a low frequency antigen in Negroes. Because of work with the Dob serum in South Africa in 1973 and 1978 this second antibody had been nicknamed 'anti-Zulu'. According to Phyllis Moores,[4] working in Natal, it had a reactivity rate of 7% in Zulus (Southern African Negroes).

The JG and Dob sera were tested in parallel and showed identical reaction patterns against blood samples from Negroes. Through the exchange of sera and appropriate red cell

‡ Reprint requests to Dr Molthan.

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samples with the Vancouver laboratory, it was determined that neither the JG serum nor several subsequent examples of anti-McC^e contained anti-Tar. The study of McC^e utilized predominantly the JG, Dob and BB sera.

Materials and methods

The 17 patients whose allo-antibodies are reported had blood samples referred to the Miller Memorial Blood Center Reference Laboratory from 1978 through 1981. Ten of the sera were from Lehigh Valley patients and seven from patients outside this area. The Dob serum was received by at least 50 other reference laboratories when it was distributed through the SCARF program.[3]

Antibody identifications were performed using commercial red cell panels[5–7] as well as in-house panels and SCARF samples.[3]

Typings for McC^a, McC^b, McC^c, McC^d, McC^e, Kn^a and Kn^b were performed using one or two drops of antisera and one drop of washed 4% saline suspension of red cells. After incubation at 37°C for 60 min an indirect antiglobulin test was performed and the results determined by macroscopic and microscopic (100 × magnification) readings. The immunoglobulin class of the antibodies was determined by re-testing the sera after treatment with 0.1 mol/l 2-mercaptoethanol. Standard techniques were used for testing the antisera against ficin pre-treated test cells, with low ionic strength saline (LISS) solutions and for antibody neutralization studies.

The antisera used to type the cells for McC^a,[8] McC^b,[9] McC^c and McC^d,[10,11] Kn^a,[12] and Kn^b,[13] had all been identified previously and their specificity confirmed. The cells were also typed for Yk^a, Cs^a, Bg^a and Bg^b by the technique described using previously identified and confirmed antisera.

The populations studied were random American White and American Negro blood donors and healthy persons involved in cases of disputed parentage. In addition to the 722 random Whites and 371 random Negroes there were an additional 94 selected unrelated individuals studied (29 McCoy/Knops Nulls, 27 McC(a−) Kn(a+), 28 McC(a + d −) and 10 McC(a + c −)).

The Amish pedigrees were available as part of a study to determine the genetics of affective disorders.[14] The remaining pedigrees included one studied to locate compatible donors for the proband and two whose probands were identified by the Blood Center while attempting to locate rare donors.

Results

The anti-McC^e antibodies were of immunoglobulin class IgG and reacted only by the indirect antiglobulin technique. Reactions were generally $+^w$ to +2 macroscopically but some, especially in Whites, were positive only on microscopic examination. The results were reproducible on in-house testing and at independent laboratories. The ability of the antibodies to agglutinate red cells was unaffected by pre-treatment of the cells with ficin, by using LISS solutions or by adding serum from antigen positive samples. The expression of the McC^e antigen varied in strength in different people partly on the basis of race (shown by the stronger expression in Negroes) and partly on the basis of the presence or absence of the other McCoy antigens: McC(a + d −) cells, for example, showed the strongest expression of McC^e. Cord blood tests demonstrated that the expression of McC^e in neonates was normal in strength and frequency, and the antigen was expressed normally on frozen de-glycerolized red cells.[15] This antibody and antigen therefore showed all the serologic characteristics of the York, Cost, McCoy, Knops system.[16,17]

Some details on the 17 producers of the anti-McC^e are shown in Table 1. The patients were all Whites who had received transfusions prior to antibody detection and all had additional allo-antibodies. Only a few were typed for other antigens in the system; the

TABLE 1
Producers of anti-McC^e

Patient	Date	System phenotype	Bg Typing	Additional allo-antibodies
HS	8/78			$-Jk^b$, $-Bg^b$
JG	4/79			$-K$
MD	7/79	Yk(a−)Cs(a−)Kn(a+) McC(a + c + d + e −)	Bg+	$-Fy^a$, −Tar
HM	7/79			$-Bg$
EMc	1/80			$-E$, $-Bg$
ES	2/80			$-Fy^a$
ER	3/80	Yk(a+)Cs(a+)Kn(a+) McC(a + c + d + e −)	Bg(a − b −)	$-K$, $-Lu^a$, $-McC^f$
EN	3/80			$-Bg^a$, $-McC^f$
EG	7/80			$-Bg$
JM	7/80			$-K$
BB	9/80	Cs(a+)McC(a + e −)	Bg(b −)	$-Bg^b$
PE	12/80	Yk(a+)Cs(a−)Kn(a+) McC(a + c + d + e −)	Bg(a−)	$-E$
SS	12/80	Yk(a−)Cs(a+)Kn(a+) McC(a + c + d + e −)	Bg(a + b −)	$-Fy^a$, $-C^w$, $-E$, $-McC^f$
ES	3/81			$-Fy^a$
DA	3/81	Yk(a+)Cs(a+)Kn(a+) McC(a + c + d + e −)	Bg(a − b −)	$-McC^f$
GS	5/81	Yk(a−)Cs(a+)Kn(a+) McC(a + c + d + e −)	Bg(a−)	$-Bg^?$, $-McC^f$
ER	7/81			$-Bg$

TABLE 2
McC^e frequencies in the American population

	Random Whites		Random Negroes	
	No.	%	No.	%
McC(e+)	71	0.0983	130	0.3504
McC(e−)	651	0.9017	241	0.6496
	722		371	
Genotypes				
McC^eMcC^e		0.0025		0.0376
McC^eMcC		0.0958		0.3128
McCMcC		0.9017		0.6496
		1.0000		1.0000

others could not be typed because their blood samples contained a mixture of their own and their donors' cells at the time of study.

Table 2 shows the frequency of McC^e in the random American White and Negro populations studied. Hardy–Weinberg calculations reveal that the antithetical antigen would occur at frequencies of 0.9975 in Whites and 0.9624 in Negroes. Table 3 shows the relationship of McC^e to the various McCoy phenotypes using the random populations and selected but unrelated individuals. Not one of the 39 McCoy/Knops nulls, nor any of the 52 McC (a−)Kn(a+) persons tested, was McC(e+). Twenty-nine consecutive random McC(a + d −) Negroes were found to be McC(e+).

Table 4 shows the phenotype frequencies based on typings for McC^a, McC^b, McC^c, McC^d, McC^e and Kn^a in 371 random American Negroes. There were 64 possible phenotypes but only 15 were observed. Table 5 shows the phenotype frequencies based on typings for McC^a, McC^c, McC^d, McC^e and Kn^a in 722 random Whites. Since McC^b does not exist in Whites 32 phenotypes were possible: 13 were observed.

TABLE 3
*Association of McC^e with the other McCoy/Knops antigens**

	Whites			Negroes		
	No.	McC(e +)	%	No.	McC(e +)	%
McCoy/Knops null (McC(a − b − c − d −)Kn(a − b −))	35	0	0	4	0	0
McC(a −)Kn(a +)	12	0	0	40	0	0
McC(a + d −)	60	36	60	29	29	100
McC(a + c −)	20	9	45	156	46	29
McC(a + c +)	705	66	9	191	84	44

*Data derived from the random populations in Tables 4 and 5 and 94 additional selected, but unrelated, persons of the phenotypes listed.

TABLE 4
*Phenotypes of random American Negroes**

McC^a	McC^b	McC^c	McC^d	McC^e	Kn^a	No.	Frequency
+	+	+	+	+	+	46	0.1240
+	0	+	+	+	+	15	0.0404
+	+	0	+	+	+	15	0.0404
+	0	0	+	+	+	24	0.0647
+	+	+	0	+	+	11	0.0296
+	0	+	0	+	+	11	0.0296
+	+	+	+	0	+	25	0.0674
+	0	+	+	0	+	81	0.2184
+	+	0	+	0	+	50	0.1348
+	0	0	+	0	+	60	0.1617
+	0	0	0	+	+	7	0.0189
0	+	0	+	0	+	21	0.0566
+	0	+	+	0	0	1	0.0027
+	0	+	0	+	0	1	0.0027
0	0	0	0	0	0	3	0.0081
						371	1.0000
McC^a							0.9353
	McC^b						0.4528
		McC^c					0.5148
			McC^d				0.9111
				McC^e			0.3504
					Kn^a		0.9865

*There are 64 possible phenotypes but only 15 were observed.

In population and/or family studies anti-McC^e failed to react with red cells positive for Bu^a, Tar, C^w, Go^a, V, VS, K, Js^a, Kp^a, Wk^a, Yt^b, Co^b, In^a, Rd, Di^a, Wr^a, Fr^a, Duch, Ri^a, Bp^a, Hey, Rb^a, Mi^a, Vw, St^a, Mt^a, Hil, Hen, Hu, M$_1$, Lu^a, Bg^a, Bg^b, Bg^c, McC^b, and Kn^b. Anti-McC^e also failed to react with Oh, −D−, K$_0$, McLeod, Jk(a − b −) Co(a − b −), Lu(a − b −), U negative, Lan negative, Jr negative, p, Rg(a −), Ch(a −), Sd(a −), Xg(a −), Yk(a −), and Cs(a −) red cells.

Studies on 51 Negro mother-children combinations showed straightforward inheritance of McC^e and lack of association with ABH, Rh, Duffy, Kidd, Lewis, MNSs, and secretor status. Population studies showed also that McC^e was unrelated to the Kell, Lutheran, P, Colton, Cartwright, Bg and Xg systems.

Figure 1 shows the Hunter pedigree. The proband has hemoglobin SS and anti-McC^d. The pedigree demonstrates the inter-relationship of the McCoy antigens as all ten McC(a + d −) members are McC(c +) and all six McC(a −) members are McC(e −). Not shown in this figure, but available on request, are data establishing that McC^e is independent of Duffy,

TABLE 5.
*Phenotypes of random American Whites**

McC^a	McC^c	McC^d	McC^e	Kn^a	No.	Frequency
+	+	+	+	+	50	0.0692
+	+	+	0	+	619	0.8573
+	+	0	+	+	15	0.0208
+	0	+	+	+	2	0.0028
+	+	0	0	+	10	0.0139
+	0	0	+	+	3	0.0042
+	0	0	0	+	2	0.0028
+	0	+	0	+	2	0.0028
+	+	+	0	0	6	0.0083
+	+	0	+	0	1	0.0014
+	0	0	0	0	1	0.0014
0	+	+	0	+	4	0.0055
0	0	0	0	0	7	0.0097
					722	1.0001
McC^a						0.9848
	McC^c					0.9765
		McC^d				0.9460
			McC^e			0.0983
				Kn^a		0.9792

*There are 32 possible phenotypes but only 13 were observed.

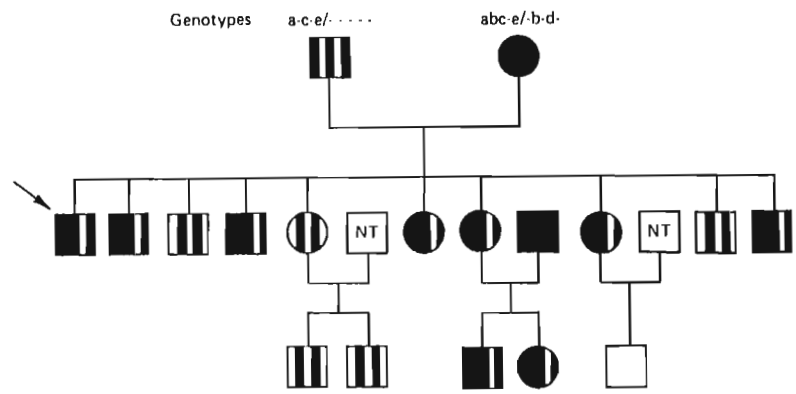

Fig. 1. M. Hunter pedigree, Negroes. Interrelationships of the McCoy antigens. ■ ● McC(a + b + c + d + e +)Kn(a +); ◧ ◐ McC(a + b + c + d − e +)Kn(a +); ◫ ◑ McC(a − b + c − d + e −)Kn(a +); ▥ McC(a + b − c + d − e +)Kn(a +); □ McC(a − b − c − d − e −)Kn(a −).

Lewis, MNSs, Ch^a and hemoglobin S. The genotype of the grandfather was considered to be McC^a _ McC^c _ McC^e/ _ _ _ _ _, and that of the grandmother McC^a McC^b McC^c _ McC^e/ _ McC^b _ McC^d _.

Figure 2 is the Amish 106 pedigree and Fig. 3 and Table 6 the Amish 110 pedigree. They demonstrate the independent inheritance of McC^e from Bg^a, Bg^b, Yk^a, Cs^a and Kn^a. Not shown but available on request are data establishing the independence of McC^e from Rh, Duffy, Kidd, P_1, MNSs, Xg^a and Se.

Figure 4 is a White pedigree demonstrating the independence of McC^e from Yk^a, Kn^a and Kn^b. Not shown, but available on request, are data which indicate that McC^e is independent of Rh, Duffy, Kidd, MNSs and P_1. This pedigree confirms that McC^e is not an alternative allele at the *Kn* locus and that Kn^b is probably not the only alternate allele of *Kn*^{a}.

Figure 5 is a White pedigree with a McCoy/Knops null parent whose brother is McC

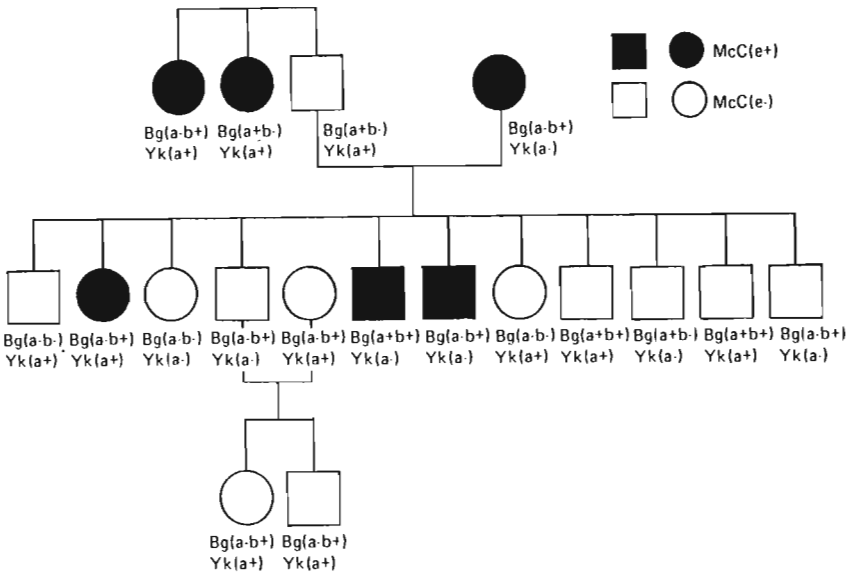

Fig. 2. Amish pedigree 106. Independence of McC^e from Bg^a, Bg^b, Yk^a. ■ ● McC(e +); □ ○ McC(e —).

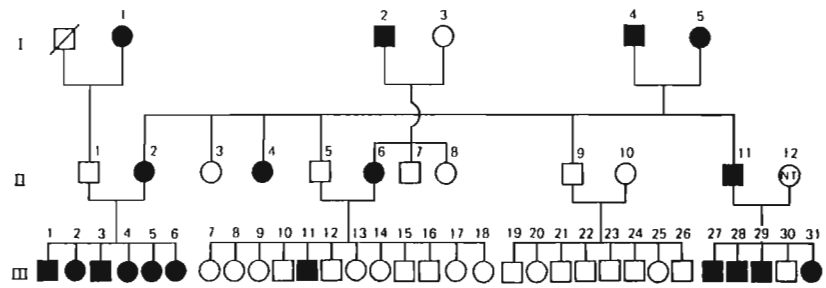

Fig. 3. Amish pedigree 110. Independence of McC^e from Bg^a, Bg^b, Yk^a, Cs^a, Kn^a (See Table 6). ■ ● McC (e +); □ ○ McC(e —).

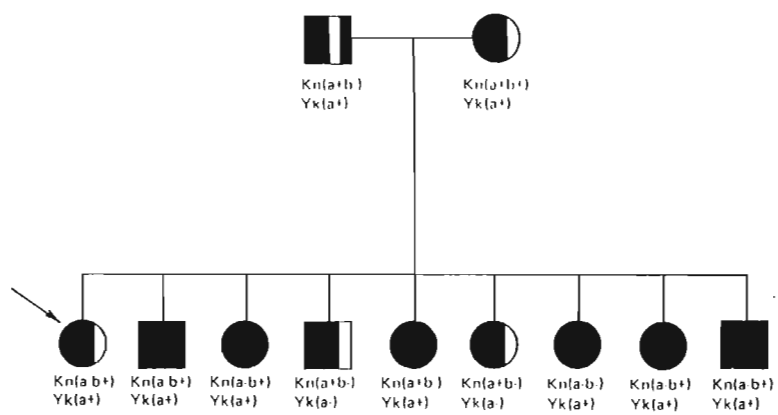

Fig. 4. Ifk. pedigree. Independence of McC^e from Yk^a, Kn^a, Kn^b. It shows that McC^e cannot be Kn^c. ■ ● McC(a + c + d + e +); ■ ● McC(a + c + d + e —); ◧ McC(a + c + d — e +).

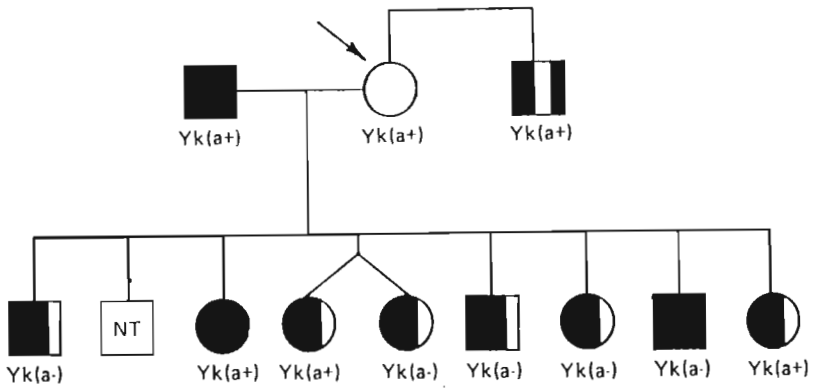

Fig. 5. Fol. pedigree. Interrelationships of the McCoy antigens. Independence of McC^e from Yk^a. (Whites) ■ McC(a + c + d + e +); ◧ McC(a + c + d − e +); ◧◐ McC(a + c + d + e −); ○ McC (a − b − c − d − e −) Kn(a −).

TABLE 6
Typings of members of the Amish 110 pedigree

I	II	III	Bg^a	Bg^b	McC^e	Yk^a	Cs^a	Kn^a
	3	9, 10, 13, 15, 17, 21, 22, 26, 30	—	—	—	+	+	+
3	1, 8	7, 12, 14	—	+	—	+	+	+
1		3, 5, 6, 29	—	+	+	+	+	+
2, 5	11	27, 31	+	—	+	+	+	+
4	2, 4	28	—	—	+	+	+	+
		20, 23, 24, 25	—	+	—	+	+	—
	7	8, 18	+	—	—	+	+	+
	6	11	+	+	+	+	+	+
	9	19	—	—	—	+	+	—
		1, 2	—	+	+	—	+	+
	5		+	—	—	+	+	—
	10		—	+	—	+	—	+
	12		—	—		+	—	+
		4	—	—	+	—	+	+
		16	+	+	—	+	+	+

(a + c + d − e +). It shows independence of McC^e from Yk^a and (not shown but available) the independence of the McC^e from Duffy, Kidd, MNSs and P_1.

In addition the five pedigrees show that McC^e cannot be the hypothetical antigens Yk^b or Cs^b.

Discussion

The initial investigation of the JG and Dob sera was limited to the testing of blood samples from Negroes. In the spring of 1980, when the frozen red cell samples of the 88 Amish were de-glycerolized for testing with anti-Bu^a, they were also tested with the Dob serum.[15] The finding of 30 'Zulu' positives amongst these samples led to the testing of random Whites and to the knowledge that this antigen occurs with a frequency of 0.0983 in that race. The antigen has also been encountered in Asiatic Indians, Puerto Ricans and in a Filipino. It has also been found[18] that seven of over 200 Shiite Arabs were McC(e+). Melanesians, who show the lowest frequencies of Yk^a, Cs^a, McC^a, and Kn^a,[17,19] have not been tested. These results confirm that McC^e is not exclusive to Negroes and that the nickname 'Zulu' is no longer appropriate.

So far the producers of the anti-McC^e have all been Whites, but there is no obvious reason why Negroes cannot make this antibody. The patients had all been transfused and had

additional allo-antibodies. Since McC^e occurs in Whites it was not necessary to suppose that the antibody producers had received donor blood from Negroes, although this was an early consideration. Only seven of the antibody producers were typed for other antigens in the system. Dob is Yk(a −), Cs(a −); SS is Yk(a −); GS is Yk(a −), and PE is Cs(a −). The significance of the finding is unclear but it may show that the McCoy antigens are more antigenic than are Yk^a and Cs^a.[19]

The realization that the new antigen was a McCoy occurred in the spring of 1980 when data for two separate presentations, one on McC^c/McC^d and one on 'Zulu', were compared. At that time it was noted that none of 21 McC(a −) Kn(a +) Negroes was 'Zulu' positive, whereas all of 13 McC(a + d −) unrelated Negroes had this antigen. Current data (Table 3) show that not one of 39 McCoy/Knops nulls, nor of 52 McC(a −) Kn(a +) persons tested was McC(e +). If McC^e was not a McCoy antigen, 14 of the Negroes and five of the Whites should have had this antigen. The finding that 29 consecutive unrelated McC(a + d −) Negroes were McC(e +) provides further evidence that the new antigen is a McCoy, for if there was no relationship only ten of these individuals would have been McC(e +). Thirty-six of 60 McC(a + d −) Whites were also McC(e +). Again, if no relationship existed only six would have had the new antigen.

The pedigrees support the belief that McC^e is a McCoy antigen. The Negro Hunter pedigree has ten McC(a + d − e +) members, five McC(a − e −) Kn(a +) members and one McCoy/Knops null. This family also demonstrates that the parents of McC(a − b +) children are not obligate McC(b +), since the null chromosome exists in the grandfather. Findings of this nature have also been encountered in other Negro pedigrees.

The Amish pedigrees[20] show the independence of McC^e from Bg^a and Bg^b, an important consideration for the frequencies of McC^e suggested that this antigen might be Bg^b in Whites and a combination of Bgs in Negroes. In pedigree 106 the mother has McC^e and Bg^b; three children inherited both antigens but four others inherited only Bg^b. In pedigree 110 there are nine persons with Bg^b and McC^e, ten with McC^e but no Bg^b, 12 with Bg^b but no McC^e and 16 who lack both antigens. Individual matings show that McC^e is not travelling with either Bg^a or Bg^b. The mating of I 4 and 5, both McC(e +), produced one probable homozygote (II 2), one heterozygote (II 11), one child of unknown zygosity (II 4), and three McC(e −) children (II 3, 5, 9). Further evidence that McC^e is not a Bg antigen is shown by Dob herself who is Bg(+), as is another antibody producer, SS. In addition, χ^2 analyses for linkage disequilibrium of McC^e/Bg give values of 0.007 to 1.4.

The Ifk pedigree (Fig. 4) shows not only that McC^e is independent of Kn^a and Kn^b but also that McC^e cannot be a third allele at the Kn locus. Considering for the moment that McC^e is Kn^c, the father's genotype would have to be either a _ _/_ _ c or a _ c/_ _ _, and the mother would have to be either a _ _/_ b _ or a b _/_ _ _. Neither genotype explains the results in the children, and there is no evidence of non-parentage in this family.

The antigen Kn^b has been described[13] as the allele to Kn^a, but the relationship is not completely straightforward. Several random McC(a +) Kn(a − b −) individuals have been observed and the frequency of Kn^b in Kn(a +) persons, reported as being 0.042–0.047, is too low. This frequency, based on Hardy–Weinberg calculations and using the incidence of 0.0111 for McC(a +) Kn(a −) (Table 5) should be 0.1028. The Ifk pedigree demonstrates two problems relative to considering *Kn*^{b} as the only allele for *Kn*^{a}: its absence in one parent and in one Kn(a −) child. The most likely explanation is a McCoy/Knops null chromosome producing an amorph at the *Kn* locus. This is similar to the situation concerning McC^b in the Hunter pedigree. The Kn^b antigen has not been detected in Negroes.

McC^e is present only when McC^a is present, but the majority of McC^a persons are McC(e −). *McC*^{e} cannot be an alternate allele at the *McC*^{c}/*McC*^{d} locus in Negroes for 0.1644 of the random samples reported in Table 4 had all three antigens, and the anti-McC^c and anti-McC^d sera were known to lack anti-McC^e. The genetic relationships of the five McCoy and two Knops antigens remain unresolved. A sixth McCoy antigen, McC^f, is currently under study.

Five of the producers of anti-McC^e also made anti-McC^f and eluates prepared from their sera are required in order to determine the frequencies of the latter antigen. There are also additional sera in the freezer whose specificities must be resolved. These, too, fail to react with all McCoy/Knops nulls. Cost and York enter into this complexity as well for they are part of the same system.[16,19] The studies which began in 1965 with Cost and York, and in 1970 with Knops and McCoy, are now revealing that these antigens belong to a very complex system.

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Paper 48

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Wd(a+) Red Blood Cells in Two Sisters of a Hei//om[1] Khoisan Family in Namibia

P. Moores[a], *E. Smart*[a], *M. Marks*[b], *M.C. Botha*[b]

[a] Natal Blood Transfusion Service, Durban; [b] Garunkuwa Hospital, Medunsa, South Africa

Key Words. Blood groups · Wd[a] factor · Low-frequency antigen · Khoisan population · African populations · Blood group genetics

Abstract. Two sisters in a Hei//om family of the southern African Khoisan race in Namibia were found to have Wd(a+) red blood cells. Wd[a] is a low-frequency antigen identified so far only in a European family in Canada and a family in Holland. The *Wd[a]* gene may have had an independent origin in the Khoisan. Alternatively, the Hei//om population may have acquired it through miscegenation.

The Wd[a], or Waldner, antigen was first reported from Canada by Lewis and Kaita [1] in 1981. The antigen was identified in members of two Schmiedeleut families living in the province of Manitoba. The Schmiedeleut are a branch of the Hutterian Brethren [2]. The Brethren, a religious, socioeconomic and communal isolate, originated as a group in central Europe in the year 1528. The Schmiedeleut branch is descended from 52 families of 215 people who migrated to South Dakota, USA, between 1874 and 1877 and later moved to Canada [2]. A family with Wd(a+) red cells in Holland has also been identified but not reported [2]. The gene determining the Wd[a] antigen is an inherited autosomal dominant [1]; anti-Wd[a] antibodies occur fairly frequently in sera containing antibodies for other low-frequency antigens [3].

The Hei//om are a population of the southern African Khoisan race. The Khoisan, physically a small people, have light yellowish-brown skin and unusual genital and other features [4, 5]. The race comprises the Khoi, who practice a pastoralist culture, and the San, who are nomadic hunter-gatherers. The Hei//om normally live in the desert on and east of the Etosha plain in Namibia [4]. In recent years, however, some bands have remained within the boundaries of the larger farms, and individuals have adopted European names through contact with the Lutheran

[1] A lateral implosive bidental sound, the nearest equivalent of which is a click.

Church. The genetic marker frequencies in the Hei//om suggest, despite their hunter-gatherer culture, that they have a Khoi rather than a San origin [4]. The frequencies in the Khoi resemble those of the Southern African Negroes more closely than those of the San [4]. The Khoi and San populations were both represented at the Cape of Good Hope when this southernmost region of Africa was first colonised in the 17th century by people of Dutch and German descent [5]. The colonists often took the Khoi women as wives, but the San either moved or were driven away.

This paper records the interesting discovery of Wd(a+) red cells in two women among 114 Hei//om who were being tested as part of an ongoing study to identify the population origin of the Dantu antigen. Possible reasons for the presence of the *Wd*^{a} gene in the Hei//om are considered.

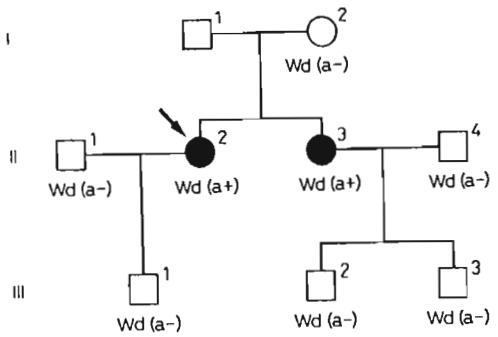

Fig. 1. Wd(a+) Hei//om family pedigree.

Materials and Methods

The blood samples were drawn into ACD anticoagulant and maintained at 2–10°C during their journey to Durban. On being received, the red cells were stored immediately in buffered glycerol-saline solution at –30°C. Aliquots were thawed when required, deglycerolysed by dialysis, washed and prepared as 2–5% suspensions in the preserving fluid of Burgess and Vos [6]. The serum used to screen the red cells for the Dantu antigen was known to contain anti-S and the following antibodies for low-frequency antigens: anti-Bp^a, -Dantu, -Hut, -Milne, -Mur, -Rb^a, -Sw^a, -Tr^a, -Wd^a and -Wr^a. These specificities were all confirmed by Dr. A. Lubenko, London, UK. The serum was used by the saline technique at room temperature (22–26°C) as the anti-S antibodies gave negative results by this method. The reagents and red cells employed locally for the Wd^a antigen study were gifts from SCARF and other colleagues. Other reagents, excluding those of anti-Rh34 specificity, were from local stocks standardised in accordance with the recommendations of the American Food and Drug Administration Bureau of Biologics; other red cells were from local sources. The anti-Rh34 sera were used unabsorbed, i.e. they were not absorbed beforehand with R_2R_2 red cells to prepare the specificity anti-hr^B. *Arachis hypogea* and *Glycine soja* lectins were included to detect possible bacterial contamination. The tests were all made by standard serological techniques, and the reagents were employed by the techniques recommended by their suppliers.

Ascertainment

One hundred and fourteen Hei//om, 107 of them members of 23 families, donated blood samples to be tested with the screening serum for the antigen Dantu. The Hei//om were all living on a farm near the town of Tsinsabis in Namibia. Strong positive results were obtained with two samples. In the subsequent studies with the reagents shown in table I, the results suggested that the antigen present in both was Wd^a. A second visit was made to Tsinsabis, where the sample donors were found to be sisters. Further blood was drawn from II-2 and members of her family (fig. 1). The sister, II-3, was unfortunately not available. All the earlier results with II-2 were confirmed, but the red cells of none of the other family members gave positive results with the screening serum.

Table 1. Results showing that the identity of the low-frequency antigen on the red cells of II-2 and II-3 was Wd^a

Reagent	Specificity	Technique	Results	
			II-2	II-3
PPM 537	anti-S (eluate)	albumin-IAT ± 22 °C	–	–
PPM 494	anti-S, -Bp^a, -Dantu, -Hut, -Milne, -Mur, -Rb^a, -Sw^a, -Tr^a, -Wd^a, -Wr^a	saline ± 22 °C	2	2
Motaung	anti-S, -Bp^a, -Wd^a	saline ± 22 °C		3
	anti-S, -Dantu, -Wd^a, -Wr^a	IAT 37 °C		2
PPM 15	anti-Dantu, -Mur or -Mi^a, -Rb^a, -Wd^a, -Wr^a	saline ± 22 °C		2
G. Stevens	anti-Bp^a, -Donaldson, -Lanthois, -Mt^a, -NFLD, -Wd^a, -Wr^a	saline 37 °C	1	1
B. Paschal	anti-Bp^a, -Rb^a, -Wr^a	LISS-IAT 37 °C	–	–
L2000	anti-Mi^a	IAT 37 °C	–	–
Devereaux	anti-Bp^a(?), -Pt^a, -Tr^a	saline ± 22 °C	–	–
PPM 642	anti-Hut	IAT 37 °C	–	–
SRJ 23-11, -80	anti-Mi^a	saline ± 22 °C	–	–
M 384	anti-S, -E, -Rb^a	IAT 37 °C		–
PPM 526	anti-Dantu	IAT 37 °C	–	–
PPM 496	anti-Wr^a	one-stage bromelin ± 22 °C		–

IAT = Indirect antiglobulin technique.

Results

The serum PPM 15 (table 1), after one absorption with the red cells of II-3, no longer agglutinated her cells. However, her cells and those previously stored from a member of the original Waldner family were agglutinated by the eluate prepared by the 56 °C heat technique from the absorbing cells.

Table 2 shows the other blood groups and antigens identified on the family members' red cells. The Rh:-34 Rh phenotypes of II-3, II-4, III-2 and III-3 were a surprise. However, their presence was a coincidence, as this phenotype was also found in 9 members of 5 other Hei//om families. However, the phenotype supported the claim [4] that the Hei//om were of Khoi rather than San origin.

Prof. M. Lewis and Dr. H. Kaita, Winnipeg, kindly tested the blood of II-2. Four sera containing multiple antibodies for low-frequency antigens including anti-Wd^a were found to agglutinate her red cells. After absorbing two of them with red cells from different known Wd(a+) donors, they no longer reacted with her red

Table 2. Red cell and Hp groups of II-2, II-3 and their family members

Number	ABO	MNSs	Rh	Kell	Lewis	Duffy	Kidd	I	Sd^a	Vel	Hp
I-2	A_{bantu}	Ms	R_1r	K-, Kp(a-)	Le(a-b+)	Fy(a-b+)	Jk(a+b-)	+	+	+	2
II-1	B	Ms	R_1r	K-							1
II-2	A_{bantu}	Ms	R_o	K-	Le(a-b+)	Fy(a+b-)	Jk(a+b+)	+		+	2-1
II-3	O	M_1s	r'^sR_o(Rh:-34)	K-, Kp(a-)	Le(a-b+)	Fy(a+b-)	Jk(a+b+)	+	+	+	2
II-4	A_1	MSs, He+	r'^sR_o(Rh:-34)	K-, Kp(a-)	Le(a-b+)	Fy(a-b-)	Jk(a+b+)	+	+	+	2-1
III-1	B	Ms	R_1r	K-							1
III-2	O	Ms	r'^sR_o(Rh:-34)	K-, Kp(a-)	Le(a-b+)	Fy(a-b-)	Jk(a-b+)	+	+	+	2
III-3	O	Ms	r'^sR_o(Rh:-34)	K-, Kp(a-)	Le(a-b+)	Fy(a-b-)	Jk(a+b+)	+	+	+	2

cells. However, her red cells gave positive results with the eluates prepared from the cells used to absorb these sera. Dr. A. Lubenko, London, also kindly tested the blood of II-2 with 96 sera having multiple antibodies for low-frequency antigens. The results with her red cells were marginally stronger than those of his control with the 23 sera which contained anti-Wd^a.

Discussion

As Wd(a+) red cells have been recorded so far only in 2 families of European origin, the *Wd^a* gene in the remote primitive Khoisan people of Africa may have arisen entirely independently. However, a more likely explanation is that the Hei//om acquired the gene through miscegenation with Europeans; the reverse is scarcely conceivable. Prior to the 1914–1918 war when Namibia was being administered by Germany, miscegenation between German men and Khoisan women is known to have been common. The Khoisan women were apparently sexually preferable and more amenable to the German men than were the Negro women. The miscegenator who had Wd(a+) red cells may have had ancestors in common with the Hutterite and Dutch families in central Europe. Nevertheless, Caucasoid features have not been identified in II-2, II-3 or their children. The hunter-gatherer culture of the Hei//om almost certainly precluded their ancestors from forming part of the settled community at the Cape of Good Hope in the 17th century.

Since this investigation began, a black donor with Wd(a+) red cells from Port Elizabeth in the western Cape region has been identified. Further studies to find Wd(a+) red cells in other African populations are clearly indicated.

Acknowledgements

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Phyllis Moores, PhD
Head, Immuno-Haematology Research Laboratory
Natal Institute of Immunology
PO Box 2356
4000 Durban (South Africa)

Paper 42

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Congenital Dyserythropoietic Anaemia (Type II) Presenting with Haemosiderosis

Arthur R. Bird[a], *Peter Jacobs*[a], *Phyllis Moores*[b]

[a] University of Cape Town Leukaemia Centre and Department of Haematology, Groote Schuur Hospital, Observatory, Cape, and
[b] Natal Blood Transfusion Service, Pinetown, Natal, South Africa

Key Words. Congenital dyserythropoietic anaemia · Iron overload

Abstract. A 39-year-old female with type II congenital dyserythropoiesis presented with iron overload. The clinical and haematologic features were an anaemia of variable severity, splenomegaly, numerous bizarre and binucleate normoblasts in the bone marrow, with prominent submembranous cisternae in the late forms, a positive Ham's acid lysis test and aberrant expression of the I and i red cell antigens. The iron overload resulted from gross ineffective erythropoiesis, with accelerated plasma iron turnover and increased absorption aggravated by inappropriate replacement therapy for past episodes of anaemia.

Introduction

Congenital dyserythropoietic anaemia (CDA) is a rare disorder, usually presenting in childhood with a chronic anaemia of mild to moderate degree and intermittent or persistent jaundice. It is characterized by ineffective erythropoiesis, with numerous dysplastic and multinucleated red cell precursors in the marrow. The liver and spleen are frequently enlarged and in adulthood symptoms and signs of haemosiderosis may develop as a result of the hyperplastic but ineffective erythropoiesis. This entity has been divided into three subgroups on the basis of clinical, morphologic, and serologic findings, although other variants have also been described [1]. The first South African example of type II CDA is reported, following a presentation in adulthood with iron overload.

Case Report

The patient is a 39-year-old white female who has a long history of fatigue, which began during childhood. Prior to her first pregnancy 16 years ago, anaemia was diagnosed and iron tablets were prescribed. Throughout her first and subsequent pregnancy she received iron supplementation. In addition, two units of blood were infused following her second delivery, although she cannot recall having excessive blood loss postpartum. Since this time she has had frequent 'tonics' for anaemia from her general practitioner.

In 1981 she was referred to Groote Schuur Hospital with a history of transient intolerance of fatty foods and feeling of general debility accompanied by nausea and fatigue. On examination she was moderately obese, there was mild jaundice, the spleen was palpable 1 cm below the left costal margin, but there was no hepatomegaly. Biochemical investigations revealed a total bilirubin of 33 μmol/l, with a conjugated value of 5 μmol/l. Serum gamma-glutamyltransferase (γ-GT), alkaline phosphatase (AP), aspartate transferase (AST), and lactic dehydrogenase (LDH) were normal, but the alanine transferase (ALT) levels were elevated (76 μ/l). Repeated biochemical investigations over the following 3 years confirmed a persistent unconjugated hyperbilirubinaemia, while the AST, ALT, and γ-GT showed periodic mild elevation.

Her full blood count showed a haemoglobin of 12.5 g/dl, red cell count 3.93×10^{12}/l, PCV 0.34 l/l, MCV 87 fl, MCH 32 pg, MCHC 36.8 g/dl, white cell count 6.9×10^{9}/l, and platelets 230×10^{9}/l. The differential count was normal and the red cell morphology was not remarked upon. Reticulocyte production index was less than 1.

Liver and splenic scans revealed an enlarged liver and spleen but absence of gallstones. A liver biopsy, performed in September 1981, showed increased iron deposition in the liver macrophages and in parenchymal cells, with minimal fatty change but absence of any fibrosis. The serum iron at that time was 31 μmol/l, total unsaturated iron binding capacity (TIBC) 41 μmol/l, resulting in 76% saturation of transferrin and a serum ferritin of 280 μg/l. The unconjugated hyperbilirubinaemia was considered to be due to haemolysis, and initial investigations showed a normal serum haptoglobin and haemoglobin electrophoresis. In January 1982, a working diagnosis of haemochromatosis with early liver damage was made and the patient was started on bimonthly venesections. After 3 months these became intermittent because of symptoms related to falling haemoglobin.

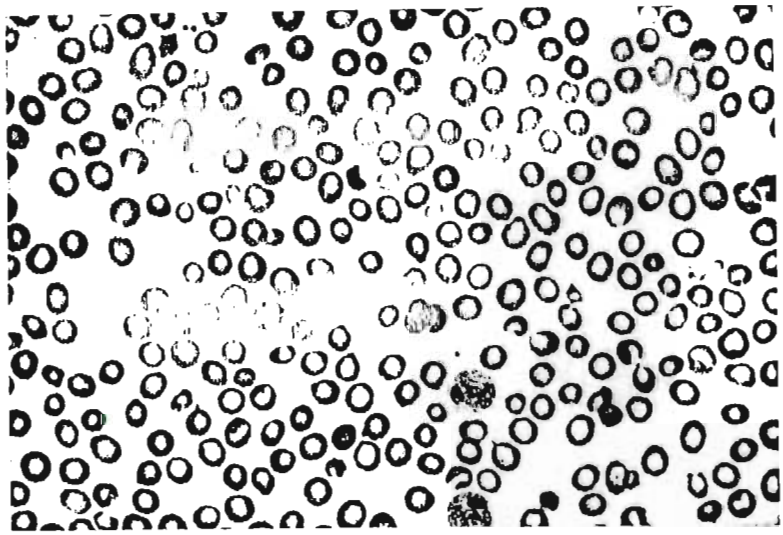

Fig. 1. Peripheral blood smear: note two hypersegmented neutrophils and a moderate degree of anisopoikilocytosis.

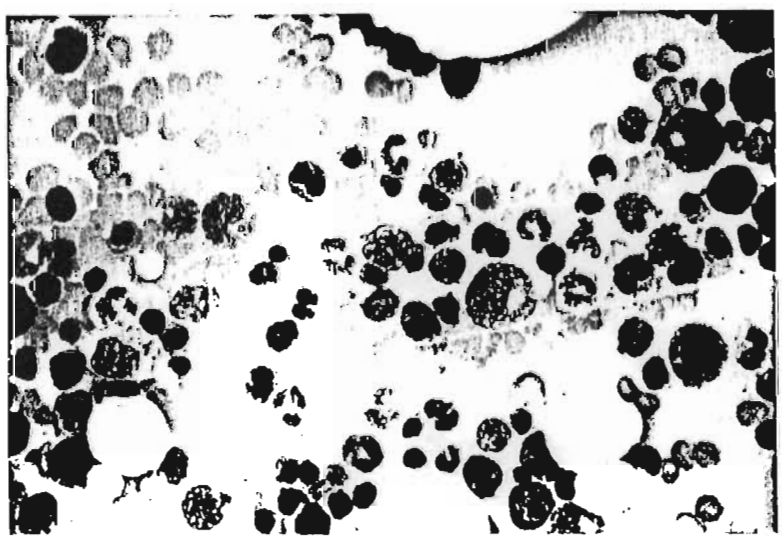

Fig. 2. Bone marrow aspiration: prominent erythroid hyperplasia with dysplasia characterized by binucleate late normoblasts.

In November 1984, the patient was reassessed. She now gave a history of abdominal discomfort and was found to be mildly jaundiced, but the liver and spleen were not palpable. Serum ferritin was greater than 500 µg/l and a venesection programme was recommenced. Bone marrow aspiration and trephine biopsy with further special investigations were undertaken to exclude the possibility of sideroblastic anaemia with an iron loading.

The *peripheral blood smear* (fig. 1) showed a normal differential count, with occasional hypersegmented neutrophils and a moderate degree of anisopoikilocytosis: vitamin B_{12} and folate levels were normal.

The *bone marrow aspirate* (fig. 2) and *trephine biopsy* showed marked erythroid hyperplasia and striking dyserythropoiesis, with binucleate late normoblasts as the most distinctive feature. Macrophages were prominent, with active phagocytosis of cells.

Ultrastructural studies (fig. 3) of the bone marrow showed prominent submembranous cisternae in several of the late normoblasts and confirmed the presence of binucleate forms.

Ferrokinetic and red cell survival studies were performed according to standard methods [2] and showed a $T^1/_2$ ^{51}Cr survival of 26.5 days (normal 25–33). $T^1/_2$ ^{59}Fe clearance was 27 min (normal 60–140) and plasma iron turnover was 3.56 mg iron/100 ml/day (normal 0.45–0.9). Red cell utilization at day 14 was 40.1% (normal > 80%).

Serological Investigations (table I)

Ham's acid lysis test was performed according to standard methodology [2] using fresh sera from 10 donors. Positive results were obtained in 3. No lysis was obtained when the patient's cells were tested against her own serum. The sucrose lysis test was nega-

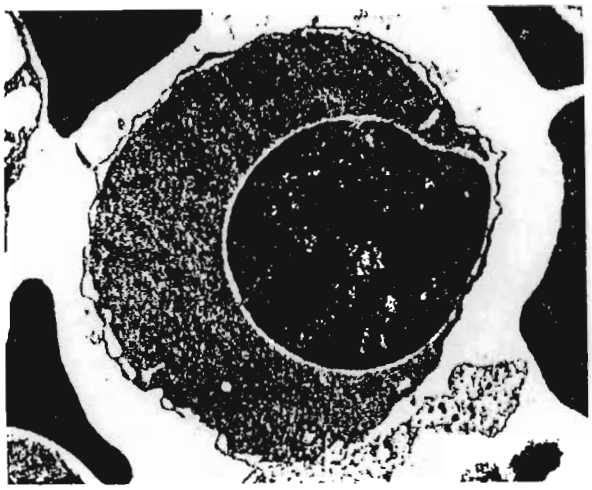

Fig. 3. Transmission electron micrograph: late normoblasts showing striking and extensive submembranous cisternae.

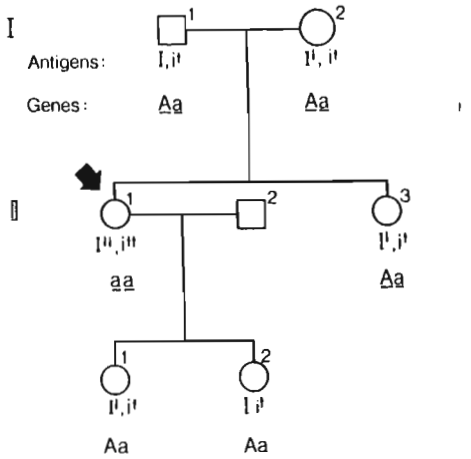

Fig. 4. Family studies: the large arrow indicates the proposita, while the small arrow indicates enhanced or reduced expression of I or i antigen on the red cells.

Table I. Seriological investigations and other family studies

	Ham's test	RBC I antigen score	RBC i antigen score	Lysis with anti-i and anti-I	Serum ferritin μg/l	Full blood count
Patient	+	15	60	+	> 500	macrocytosis
Mother	–	18	25	–		normal
Father	–	49	22	–	110	normal
Sister	–	12	30	–	55	normal
Daughter 1	–	28	10	–	50	normal
Daughter 2	–	38	4	–	80	normal

(a) Normal adult RBC I antigen score: 32–58; normal cord RBC I antigen score: 0–2.
(b) Normal adult RBC i antigen score: 0–7; normal cord RBC i antigen score: 57–68.

tive. Typical of type II CDA, her red cells had decreased expression of I antigen and markedly increased expression of i antigen, scored according to the criteria of Race and Sanger [3], in comparison with normal control red cell I and i antigens. The strength of the i antigen approximated that of cord red cells. In tests with anti-I and anti-i in the presence of complement, lysis of the red cells was observed.

Family Studies (table I, fig. 4)

Both parents and her daughters have normal full blood counts and iron stores, determined by serum ferritin levels. The red cells, however, showed mildly disturbed I and i expression. Phenotyping at the major histocompatibility complex showed that the patient and her eldest daughter share two HLA haplotypes while the mother has an A3:B7 haplotype. This latter phenotype has been linked with haemochromatosis, although the A3:B14 haplotype is the most frequent association [4].

Discussion

This case report is of interest for three reasons. Firstly, this is apparently the initial report of a classic type II CDA in a South African adult. We have previously encountered a child with morphologic features of type II CDA but without any of the other serologic or haematologic associations [5]. In addition, we have studied a patient with ineffective erythropoiesis and a shunt hyperbilirubinaemia, where dysplasia of the red cell precursors was absent.

Secondly, the clinical presentation of this patient with features of iron overload is later than usually described. In a series of 39 patients described by Ver-

wilghen et al. [6], 35 presented before the age of 20. The commonest clinical feature was jaundice, while haemosiderosis was reported in 7 patients. Anaemia (haemoglobin < 11 g/dl) was seen in 27. The haemoglobin level in our patient has generally remained within normal limits. The anaemia she experienced during pregnancy may have resulted from secondary folate deficiency brought on by the extra nutritional requirements of pregnancy. Regrettably, no records of her red cell folate levels during pregnancy are available. Secondary haemochromatosis has been reported as a complication of CDA [1], although the factors responsible for iron loading in these disorders have not been clearly defined. One hypothesis [7] is that increased plasma iron turnover may be an important factor facilitating enhanced iron absorption. In this patient there is the additional source of overload following the ingestion of iron-containing medications. However, secondary haemochromatosis may occur without prior iron therapy or blood transfusion. Cazzola et al. [8] studied 8 patients with CDA who had received no medicinal iron and had insignificant red cell transfusions during the course of their illness. All the patients had increased iron stores, including severe overload in 2, with evidence of parenchymal organ dysfunction. Iron loading was dependent on the degree of anaemia, but was closely related to age and total erythropoietic activity was increased.

Finally, the family study is interesting. The red cells of the father and mother (who are unrelated), sister and one daughter have increased i antigen expression, while the red cells of the mother, sister and same daughter also have decreased I antigen expression. Negative Ham's tests were registered and no clinical or other serological abnormalities were detected. The findings may be explained by supposing that the proposita has inherited a double dose of recessive gene [6] and that this codes for type II CDA. In addition, the variable strength of the I and i antigens of the red cells in the other family members show that the gene is capable of expressing itself partially when heterozygous.

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Prof. Peter Jacobs
University of Cape Town Medical School
Department of Haematology
Anzio Road
Observatory 7925, Cape (South Africa)

CHAPTER VIII

BLOOD GROUP CHIMERAS

VIII.1 PAPERS AND STUDIES

Blood chimerism in a pair of twins
Paper 4 by Booth, Plaut, Jamie, Ikin, Moores, Sanger and Race

Blood group and tissue mosaicism in a Natal Indian woman
Paper 22 by Moores

Durban II dispermic chimaera
Paper 30 by Moores

Zulu XX/XX dispermic chimaera from Natal with two populations of red blood cells and patchy skin pigmentation
Paper 43 by Moores, Watkins, Greenwell, Dunn and Bird

Pigment anomalies of the skin in the human chimaera: their relation to systematized naevi
Paper 36 by Findlay and Moores

VIII.2 INTRODUCTION

Two types of blood group chimera are known: twin and dispermic [Race and Sanger, 1975, p511-546]. In the twin type, early tissue cells which will eventually become erythropoietic tissue are believed to escape from one twin (the donor) of a non-identical twin pair into the other twin (the host) through an anastamosis which forms unexpectedly between adjacent blood vessels in their placentas. The cells find their way through the host twin's circulation to the erythropoietic tissue, where they settle down. When this tissue matures, it produces the blood cells of both twins. The host twin tolerates the donor twin's cells because the anastamosis formed before the immune system was sufficiently mature to recognise that they were foreign. The blood cells of both twins are present in the host twin's circulation throughout life. The first example of twin chimerism was identified in 1953 by Dunsford, Bowley, Hutchinson, Thompson, Sanger and Race. The chimera was a woman whose twin had died in infancy. Sixty percent of her red cells were group O, k, Jk(a+b+) and 40% group A_1, Kk, Jk(a-b+). As the chimera had H but no A substance in her saliva, the O cell line was presumed to be her own.

In the dispermic type of chimera, double fertilization is believed to have occurred [Race and Sanger, 1975, p536]. The chimera's mother may contribute a single egg, which subsequently divides into two, a single egg with an unexpectedly retained polar body or two dissimilar eggs. The chimera's father contributes two dissimilar sperms. After the mothers contribution has been fertilized, for unknown reasons and at an unknown stage the two zygotes (or their subsequent cell morulas) merge. All further development then continues as a single individual. The tissues in dispermic chimeras are a mosaic of those which would have formed the same tissues in a pair of dissimilar twins. The first dispermic chimera was reported in 1962 by Gartler, Waxman and Giblett and in 1963 by Giblett, Gartler and Waxman. The chimera was an XX/XY hermaphrodite with 50% MS, R_1R_2 and 50% MNs, R_1r red cells. One eye was hazel in colour and the other eye brown.

VIII.3 COMMENTARY

VIII.3.1 Attainments in London, 1955 to 1957

VIII.3.1.1 Twin chimeras

In 1957, the authors of paper 4 reported a case of twin chimerism in which, this time, both twins were alive. The propositi were a brother and a sister, both of whom had two populations of red cells. The brother had 86% group A and 14% group O and the sister 1% group A and 99% group O red cells. The group A cells were type A_1, MSs, R_1r, Fy(a-b+), Jk(a+b+) and the group O cells type MS, R_2r, Fy(a+b+), Jk(a+b-). The brother secreted A and H substances in his saliva and his sister secreted H substances. This suggested that the group A red cell line belonged to the brother and the group O red cell line to the sister. The authors described in detail the method they had used to separate the two red cell populations. Briefly, *Dolichos biflorus* anti-A_1 lectin had been added to a sample of washed red cells to agglutinate those that were group A. The agglutinates were carefully separated from the unagglutinated red cells and deagglutinated by adding commercial A substance.

Another example of dispermic chimerism, in which again both twins were alive, was reported in the same issue of the journal in 1957 by Nicholas, Jenkins and Marsh.

VIII.3.2 Attainments in Durban, 1961 to 1991
VIII.3.2.1 Dispermic chimeras
VIII.3.2.1.1 Durban I

Mrs T.R. was the first known XX/XX dispermic chimera [paper 22]. A Tamil-speaking Indian woman, she was identified when her blood sample, received for antenatal studies, gave a weak positive result with an incomplete anti-D reagent during tests made to determine her Rh phenotype. When her blood was re-examined for confirmation with further anti-D reagents, it was found to contain a mixture of approximately 50% agglutinated and 50% unagglutinated red cells. As mixed-field results had also been detected with anti-A, both *Dolichos biflorus* anti-A_1 lectin and incomplete anti-D were used independently to separate the two populations. The subsequent studies showed that 55% of the chimera's red cells were group B, MNs, R_1r, Fy(a+b-) and 45% group A_1B, MNSs, rr, Fy(a+b+). Surprisingly, both populations were Lewis type Le(a+b-), yet the chimera secreted B and H substances! As in the XX/XY dispermic chimera from Detroit [Zuelzer, Beattie and Reisman, 1964; Beattie, Zuelzer, McGuire and Cohen, 1964], however, her non-secretor tissue was probably producing enough Le^a substance to "coat" both populations. At the chimera's conception, her mother was believed to have contributed *B* and *O*, and *Se* and *se*, genes. Her father's contribution was almost certainly A^1 and *se* genes and an X chromosome in one sperm and *O* and *Se* genes and an X chromosome in the other. In addition, the chimera had an excess of HLA antigens on her lymphocytes. In all the tests considered to have given positive results, only 50% of her lymphocytes had reacted with the anti-HLA reagents. Mrs T.R. had borne four children, two of whom had group B and two group O red cells. Over large parts of her legs and the greater part of her torso, she had multiple small patches and striations of light-brown and dark-brown skin pigmentation.

VIII.3.2.1.2 Durban II

[Quoted from Blood Groups in Man, by R.R. Race and R. Sanger, by kind permission of Blackwell Scientific Publications Ltd, Osney Mead, Oxford, England].

A Black baby girl who had a male twin was found to have two populations of red cells, but their proportions in her blood were not estimated [paper 30]. When separated, their groups were seen to be B, MNS, Fy(a+b+) and O, MNs, Fy(a-b+). Her male twin, who had group A, MNSs, Fy(a+b+) red cells, could not have supplied either of his twin sister's red cell lines. Karyotyping showed that his sister's lymphocytes carried only XX chromosomes: she was therefore another XX/XX dispermic chimera. The case was a classic example of the inadvisability of assuming twin chimerism merely because a twin was present.

VIII.3.2.1.3 Durban III

Mrs O.M. was an Black woman. She was the third dispermic chimera with two populations of red cells and a normal XX karyotype identified in Durban [paper 43]. One other dispermic chimera with a similar karyotype has been recorded, but her blood contained only a single population of red cells [Mayr, Pausch and Schnedl, 1979]. The Durban III chimera was found when she gave a blood donation. In the subsequent routine tests to determine her ABO phenotype, her red cells were grouped as O, but no evidence of the corresponding A,B antibodies expected in her serum was detected. Further studies showed that her blood contained 99% group O and 1% group A_2B red cells. In the red cell enzyme studies, her group O red cells were identified as acid phosphatase type BA and peptide-A type 8-2 and her group A_2B cells as acid phosphatase type RA and peptide-A type 1. The chimera was a non-secretor of ABH and Lewis substances. H, A and B transferases were identified in her serum. The levels of her A and B transferases were such that tissues other than her haemopoeitic tissue had to be carrying her genetically A^2B cell line and contributing these enzymes to her plasma. At her conception, her mother was

believed to have contributed A^2 and *O* genes and her father two sperms, one carrying a *B* gene and an X chromosome and the other an *O* gene and an X chromosome. Moreover, the chimera had very large patches of light-brown and dark-brown skin pigmentation on both the front and back of her thorax and arms.

VIII.3.2.1.4 Patchy skin pigmentation

The interesting skin pigmentation patterns in the Durban I and Durban III dispermic chimeras and in some XX/XY dispermic chimeras recorded elsewhere was the subject of Paper 36. The presence of the products of two zygotes had clearly disturbed it. The skin of Durban I had a generally mottled appearance; the mottled areas were both larger and darker on her right than her left side and darker where friction or exposure to sunlight had occurred. The pattern resembled the epidermal naevi associated with the so-called naevus lines of Blaschko. By contrast, the skin of Durban III, and to some extent that of the XX/XY chimeras discussed, carried large box-like blocks of dark-brown pigment with fairly straight edges that appeared to overlie a more general light-brown-pigmented background. The pigment on the right side of Durban III was darker than on her left side, both in front and behind. It would have been attractive if one simple scheme accounted for the pigment patterns in chimeric, naevoid and other genetic states. The stem cell, organ system, tissue layer and cell mixture, however, were all rival possibilities, and the processes of induction, threshold phenomena and action at a distance had all to be considered. The somatic mesoderm and neural crest contributions to the connective tissue might cause stepwise demands for the mixed pigment induced in the epidermis by the two populations of cells. Ordinary melanoblast clone migration as the answer was nevertheless too simple. The size of the pigmented patch and its colour was more likely to be controlled by a far more elaborate process. The level of skin colour in the epidermis might merely be induced by the composition of the underlying connective tissue.

SUMMARY

Durban I

Approx. 50% B and 50% A_1B red cells

Group B red cells

MNss R_1r (DCe/dce)
Le(a+b-) Fy(a+b-)
AcP:AB GPT:2 AK:1
EsD:2

Group A_1B red cells

MNSs rr (dce/dce)
Le(a+b-) Fy(a+b+)
AcP:A GPT:2-1
AK:2-1 EsD:2-1

Leucocyte antigens

HL-A1 A10 W28
HL-A8 A13(+) W5

Durban II

Group B and group O red cells; percentages not determined

Group B red cells

MNS Fy(a+b+)

Group O red cells

MNs Fy(a-b+)

Durban III

99% group O and 1% group A_2B red cells

Group O red cells

AcP:AB Pep-A:8-2

Group A_2B redcells

AcP:AR Pep-A:1-1

BLOOD CHIMERISM IN A PAIR OF TWINS

BY

P. B. BOOTH, M.R.C.S., L.R.C.P.

GERTRUDE PLAUT, M.D.

J. D. JAMES, M.R.C.S., L.R.C.P.

North London Blood Transfusion Centre, Edgware, Middlesex

ELIZABETH W. IKIN, B.Sc.

Medical Research Council Blood Group Reference Laboratory, the Lister Institute, London

PHYLLIS MOORES, B.Sc.

RUTH SANGER, Ph.D.

AND

R. R. RACE, Ph.D., M.R.C.S., F.R.S.

Medical Research Council Blood Group Research Unit, the Lister Institute, London

This paper describes some tests on the blood of a brother and sister who are twins: the blood groups show that each twin is living on red cells only some of whose ancestors were directly inherited, the rest having been acquired as grafts *in utero* from the opposite twin; the presence of female "drumstick" nodules on the nuclei of some of the polymorphs of the male twin shows that ancestral white cells too must have been successfully grafted. Mixed blood in dizygotic twins is common in cattle but appears to be extremely rare in man.

In 1916 Lillie demonstrated an anastomosis of blood vessels between the chorions of dissimilar bovine twin embryos. Lillie said that the rapidly elongating embryos "meet and fuse in the small body of the uterus at some time between the 10 mm. and 20 mm. stage. The blood vessels from each side then anastomose in the connecting part of the chorion; a particularly wide arterial anastomosis develops, so that either foetus can be injected from the other." Lillie realized that in this anastomosis was to be found the explanation of the freemartin condition: hormones of the male twin, having crossed the anastomoses, could divert the proper development of the sex apparatus of the female twin. This explanation, so far as we know, has not been superseded.

In 1945 Owen discovered that the red cells of dizygotic twin cattle were antigenically a mixture of two kinds and that this could be explained if primordial red cells, having crossed the anastomoses, could take root and grow in the opposite twin. This discovery was of fundamental importance to the theory of the nature of antibodies (Burnet and Fenner, 1949).

In 1953 a blood donor, Mrs. McK., was found to have two kinds of blood (Dunsford, Bowley, Hutchison, Thompson, Sanger and Race, 1953). Of her red cells 60% were O, *kk*, Jk^aJk^b and 40% were A_1, *Kk*, Jk^bJk^b. On inquiry Mrs. McK. said she had had a twin brother who died in infancy 25 years before. As Mrs. McK. secreted H substance but no A substance in her saliva it was assumed that the O cells were her own and that the A cells were descendants of immigrant embryonic cells from her brother. Mrs. McK. is normal in every way and is clearly not a freemartin, for she has now had three children. Certain physical properties of the anti-B of Mrs. McK. showed it to be of the kind found in persons of the genotype A_1O, as opposed to the kind found in persons of the genotype *OO* (Filitti-Wurmser and Jacquot-Armand, 1956).

Five months after the publication of this human example, mixed blood was reported in a pair of sheep twins (Stormont, Weir, and Lane, 1953); the twins were of different sex, and the female was found to be a freemartin.

In their masterly work "Quantitative Studies on Tissue Immunity," Billingham, Brent, and Medawar (1956) describe the finding that twin chicks are chimeras —probably all of them.

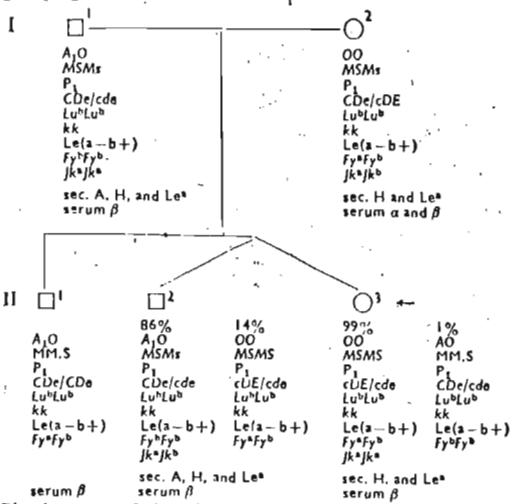
Another example of chimerism in man is reported by Nicholas, Jenkins, and Marsh (1957) in the present issue of this *Journal*.

Note.—In a letter to the *British Medical Journal* in 1953 Mr. S. Baron, of California, said that he himself must be a chimera because in his R.A.F. days he had been told that he was group "AO." Curiously, the grouping had been done by two of us (E. W. I. and R. R. R.), and reference to our records showed him to be a normal group B. We mention this in the hope that the letter will no more be quoted as another example of a chimera.

Blood Groups of the Family W.

Recently Miss W., aged 21, made her first donation of blood at the North London Blood Transfusion Centre. Her red cells appeared at first to be a very weak form of group A, for when mixed with anti-A or anti-A+B on a slide and looked at through the microscope very small agglutinates were seen which were vastly outnumbered by unagglutinated cells: anti-B caused no agglutination. The serum of Miss W. contained anti-B but no anti-A. The very weak reaction with anti-A led to the testing of the blood of the W. family and the realization that we were dealing with another example of chimerism, this time with the advantage that both twins were alive.

The blood groups of the family of Miss W. are shown in the pedigree (see Fig.). Nothing unusual was found in the groups of her father or her mother or her elder brother. When the twin brother's blood was tested it was easily seen to be a mixture, because he has a much less unequal proportion of the two components than his sister. The two components of the mixed blood possessed by the twins differ in five systems: ABO, MNSs, Rh, Duffy, and Kidd. The grouping of the twin brother's blood is described first.



Blood groups of the W. family. The groups directly under the twin chimeras, II-2 and II-3, are those of their inherited blood; a little to the right are those of their grafted blood. Antisera used: anti-A, -A_1, -B, -A+B, -H; anti-M_1, -N, -S, -s, -U, -Mia, -Vw; anti-P+P_1, P_1; anti-C, -c, -Cw, -D, -E, -e, -f, -V; anti-Lua, -Lub; anti-K, -k; anti-Lea, -Leb; anti-Fya; anti-Jka, -Jkb. Arrow = propositus.

Method of Separating O and A_1

The original method used by two of us (R. S. and R. R. R.) to separate the two components, O and A_1, in the blood of Mrs. McK. was used again. The method is simple in principle. Anti-A is added to the mixed blood in a Petri dish. The larger agglutinates of A cells are collected and are then freed from agglutinin by shaking in the presence of dissolved A substance. The remaining mixture of A and O cells is put into a long tube and left to settle; the agglutinated A cells sink more quickly than the free O cells, which are taken from the supernatant fluid.

As the method has not previously been published it may be useful to give some details here—though doubtless improvements could be made to every stage.

The amount of blood required depends on the proportions of the components. For II-2 three Petri dishes were enough, but for II-3 we used ten before we had a really adequate sample of A cells—for only about 1% of her cells were A.

For the separations we used human anti-A and an extract of the seeds of *Dolichos biflorus*—which agglutinates A_1 cells more avidly than does human anti-A (Bird, 1951). We are grateful to Lieutenant-Colonel G. W. G. Bird, who suggested the use of the extract because of its avidity and because, being a simpler substance than human anti-A, it might later be more easily won from the agglutinated A cells by the A substance. This proved indeed to be true.

Probably the more dilute the extract the better, so long as it can still cause enormous agglutinates of A_1 cells on a slide: a crude extract used diluted 1 in 5 was found to sensitize A_1 and O cells for antiglobulin serum, so that subsequent tests had to be on saline suspensions of the separated cells. This difficulty was later overcome by using an extract that had been purified by chemical means: it could probably have been overcome by mere dilution of the extract.

1. Into each Petri dish put 2 ml. of diluted *Dolichos biflorus* extract (or 2 ml. of human anti-A diluted 1 in 2) and 0.5 ml. of a 50% suspension of the A_1:O mixture of cells. Rock until the agglutinates do not seem to be getting bigger. (If the A cells are relatively few the agglutinates are very small and difficult to catch: the agglutinates can be made larger by centrifuging the mixture and returning it to the dish.) Pipette the larger agglutinates into a tube marked A. Rock the dishes again and pipette their contents into a tube marked O; the dishes are washed with a few millilitres of saline, which is transferred to tube O.

2. Fill tube A with saline and invert: when the agglutinates have settled (in a few seconds if they are large ones) remove the supernatant saline; wash in this way three more times. To the deposit of agglutinates add 3 ml. of A substance (Knickerbocker) and 3 ml. of saline. Shake hard and leave for about 20 minutes. Look for agglutinates in the supernatant suspension: if they are present add more A substance or perhaps wait longer. Fill the tube with saline, leave it to stand, first on a slope then vertically, for about an hour. Take as much as needed of the supernatant suspension, and wash the cells therein three times: this is the A sample.

3. While 2 is going on centrifuge tube O to bring small agglutinates together, but do not remove the supernatant antibody and saline. Add more saline, if necessary, to fill the tube; invert. Stand, first on a slope then vertically, for about an hour. Remove sufficient supernatant suspension, and wash the cells therein three times: this is the O sample.

Groups of the Twin Brother, II-2

The addition of anti-A caused large agglutinates to appear, but even by the naked eye a pink background could be seen which the microscope showed to be due to very large numbers of unagglutinated cells.

Some of the results of testing the red cells of II-2, untreated and after separation of the A and O components, are shown in the Table. The groups of the two fractions are given in the pedigree.

Reactions of Certain Critical Antisera with the Red Cells of II-2 Before and After Separation of the Two Components

Red Cells of II-2	Anti						
	−A	$-A_1$	−B	−s	−C	−E	$-Fy^a$
Untreated ..	+++*	+++*	−	w	+++*	+*	w*
A separated	+++	+++	−	+	+++	−	−
O "	−	−	−	−	−	+++	++

The asterisk means that unagglutinated cells were very obvious.

Theoretically the separation could have been made with anti-C, with anti-E, with anti-s, with anti-Fy^a, or with anti-Jk^b, but the relative weakness of these agglutinations and the scarcity of the antisera made the separation with anti-A the obvious choice.

We did, however, make one cross-check. The red cells of II-2 were separated with anti-E (lacking incomplete anti-D) made very avid by the addition of Löw's (1955) papain solution. The separation was successful, for the supernatant unagglutinated cells were *CDe/cde*, and, as anticipated, they were group A. (They were strongly agglutinated by anti-A and practically no unagglutinated cells were to be seen—in marked contrast to the effect of anti-A on the untreated sample.) The E cells were separated by mixing the blood of II-2 with the same anti-E but without the papain solution. The collected agglutinates could be freed from antibody by simply shaking hard with cold saline. Tests showed these cells to be *cDE/cde*, and, as expected, group O. (The cells could not be freed from anti-E when papain had been used.)

Groups of the Twin Sister, II-3

The addition of anti-A caused very small agglutinates to appear, but the vast majority of the red cells were group O. The A cells were evidently too few in number to cause any difficulty in grouping by the other antisera, all of which are less sensitive than anti-A. The testing of the O cells was therefore straightforward, and, as expected, the groups were those of the O component in the blood of the twin brother. The separation of the A cells was rather laborious, but it was done by the method described above. The groups of both fractions are shown in the pedigree.

Proportions in the Mixtures

Counts of the proportion of the two kinds of red cells possessed by each twin were kindly made by Dr. P. L. Mollison and Miss Marie Cutbush, of the Medical Research Council Blood Transfusion Research Unit. Several ingenious methods were used and the results were:

Twin brother, II-2	..	A 86%	..	O 14%
Twin sister, II-3	..	A 1%	..	O 99%

Genotypes of the Twins

Both twins are secretors: the brother secretes in his saliva A and H (and Le^a), the sister secretes H (and Le^a). This would seem to establish beyond doubt that the genetic groups of the boy twin are those of the A series and of the girl twin those of the O series. The saliva of the boy twin did not differ detectably from that of his father in the amount of A and H substance it contained.

White Cells of the Twins

Dr. W. M. Davidson has kindly examined films of the blood of the twins and of their parents. In the films of the male twin polymorphs were found with the "drumstick" nuclear knobs present normally only in females (Davidson and Smith, 1954). The proportion of cells with "drumsticks" was compatible with a mixture of male and female blood in the proportion shown by the blood groups.

Discussion

Ninety per cent. of dizygotic bovine twins are said to have mixed blood, but in man it must be a great rarity. Were it not so the Transfusion Services would have detected more ABO mixtures, for, in England, about 34% of dizygotic twin pairs differ in their reactions with anti-A or with anti-B. (This figure is extracted from Smith and Penrose, 1955, Appendix Table 2, which gives phenotypic sib-sib frequencies for the ABO system in England.) The Blood Group Research Unit has tested, without finding a mixture, samples of blood from 77 pairs of twins thought by skilled observers to be dizygotic: 68 of them differed in their ABO, MNS, or Rh groups, and the antisera of these three systems should have been able to detect mixtures, had they occurred, in one or the other twin. (Of the remaining nine pairs, seven differed on other systems.)

It follows that anastomoses between dizygotic human twins either must be very rare or must develop at a time when the ancestral blood cells are losing their faculty to take root in a foreign bed, or when the host tissue is becoming less tolerant of immigrant cells. Professor J. D. Boyd thinks that anastomoses are rare but probably not as rare as the very infrequent recognition of chimeras would suggest. In monozygotic twins, on the other hand, mixed blood must be very common—though undemonstrable.

Judging by the W. twins it seems that, in man, the proportion of the two kinds of blood in one twin is independent of the proportion in the other twin. This is not what happens in cattle, for according to Irwin (1955) each of a pair usually has the same mixture; that is to say, if twin A has 60% A blood and 40% B blood then his fellow twin B will also have 60% A blood and 40% B blood.

Dr. Davidson's finding of female "drumsticks" in the nuclei of some of the polymorphs of the male twin shows that the ancestors of these cells too can be successfully grafted. The proportion of polymorphs with "drumsticks" was compatible with a graft of the extent shown by the red cells; if this proves a general rule it may have useful implications in the study of the lineage of these cells.

The female twin is of the genotype *OO*, and 99% of her red cells are group O. Her serum contains anti-B but no anti-A. The absence of anti-A is presumably a manifestation of the phenomenon of acquired tolerance, studied particularly by Billingham, Brent, and Medawar (1953, 1956); the presence of A cells early in uterine life has presumably inhibited the production of anti-A.

In cattle and in fowls the tolerance to foreign blood cells acquired in foetal life can later be shown not to be limited to blood cells: the majority of dizygotic twin cattle are fully tolerant to grafts of each other's skin (Anderson, Billingham, Lampkin, and Medawar, 1951), and the same is true of chickens (Billingham *et al.*, 1956). Professor Medawar and his colleagues think it probable that skin could be successfully grafted between Miss W. and her twin brother.

Both twins are secretors. They secrete the ABH antigens for which they have inherited genes: they do not secrete the antigens of their grafted red cells. From this we may assume that the ABO genes do their parallel work of manufacturing water-soluble A, B, and H substance in cells other than those in which they are busy providing alcohol-soluble A, B, and H substance for the red cells.

The first human chimera to be recognized, Mrs. McK, has demonstrated conclusively, by having three children, that the hormones of a human male foetus had no power to upset the normal sexual development of his female twin. In the parallel situation a cow is sterile. Evidently the human male is backward, compared with the bull, in developing his endocrines—which is perhaps not surprising considering the relative tempo of their two lives. In man we suppose that the embryonic female hormones have no effect on the development of the male twin—but on this point there is so far no evidence.

Summary

Two human blood chimeras are described—twins of different sex. The male twin has 86% A_1 and 14% O red cells; he also has female "drumsticks" in some of his polymorphonuclear white cells. The female twin has 99% O cells and 1% A cells.

The A_1 cells are *MSMs, CDe/cde, Fy^bFy^b, Jk^aJk^b*; the O cells are *MSMS, cDE/cde, Fy^aFy^b, Jk^aJk^a*; in their other groups they do not differ. Secretion tests on saliva show that the A_1 series belongs genetically to the male twin and the O series to the female.

We are grateful to the blood donor, Miss W., and to her family, who have been most generous in their help. We are indebted to Dr. Amos Cahan, of the Knickerbocker Foundation, New York, for a large supply of A substance.

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Paper 22

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Blood Group and Tissue Mosaicism in a Natal Indian Woman

PHYLLIS MOORES

Natal Blood Transfusion Service, Durban

Abstract. A normal 46, XX karyotype was demonstrated in an Indian woman who has 2 approximately equal populations of red cells and of leucocytes. She also has skin mosaicism. She is a normal female and has borne 4 healthy children. The ABO, Ss, Rh and Fy groups of her 2 red cell populations differed, but both were Le(a+b–) although she is a secretor of B and H substances. The relationship between this and the 7 examples of dispermy with blood group mosaicism reported in the literature is discussed briefly.

Key Words
Blood group mosaic
Dispermy
Karyotype
Mosaicism
Tissue mosaic

The condition of dispermy was first recognised in man in 1962 by GARTLER *et al.* [8] in an XX/XY hermaphrodite who had blood group mosaicism. Since then 6 further cases have been recorded; 2 of them with additional skin mosaicism [1, 5, 11, 16]. Five were Caucasians [3–5, 7, 10, 11, 14] and one was a Negro/Amerindian/Caucasian [1, 16]. Some evidence of hermaphroditism and XX/XY karyotypes was present in 6 of the cases [1, 4, 5, 7–11, 14, 16]; and one, a male mongol, was a mosaic for trisomy 21 [3]. Only the original case was orientated as a female: the 2-year-old white girl of GARTLER *et al.* [8, 9]. Dispermy is believed to be the result of separate fertilization of one or more egg nuclei by 2 sperm, but the exact nature of the female contribution, and when and how the products of fertilization unite to develop into a single individual, is uncertain.

In this paper details are given of an eighth case of dispermy encountered in 1965 [13] when an Indian woman was found to have blood group and tissue mosaicism. She was a normal female with an XX karyotype,

and had borne four normal healthy children. A preliminary account of her blood groups was included in the review by RACE and SANGER [15].

Case History

When first seen the Indian woman, Mrs. T. R., who was a member of the Tamil sect of the Dravidian Indians, was 6 months pregnant with her fourth child. She was in good health and had come to the antenatal clinic for a routine examination. Mixed fields of agglutinated and unagglutinated red cells were seen in tests with anti-D. No history of a recent blood transfusion or bone marrow grafting was elicited, and the ABO group of her fourth child at birth excluded the possibility of feto-maternal transplacental haemorrhage (fig. 1). Dispermic mosaicism, as opposed to twin chimerism, was suspected when Mrs. T. R.'s mother stated that there had been no twin or unexplained tissue at the birth of her daughter. Blood group tests on several occasions during the following 6 years, when Mrs. T. R. was not pregnant, confirmed the persistence of the 2 populations of red cells.

Results

Red cell studies. In addition to the unexpected mixed agglutination with anti-D, further evidence of the presence of more than one type of red cell was obtained when Mrs. T. R.'s cells were grouped with anti-A, anti-C, anti-S and anti-Fy^b. In each test approximately 50% of the cells were agglutinated. Two distinct red cell populations were separated by using anti-A serum [2], and on a later occasion with incomplete anti-D serum followed by antiglobulin reagent. The groups of the separated cells were as follows: (1) A_1B, MNSs, P_2, Tj(a+), cde/cde, Lu(a–b+), kk Kp(a–b+) Js(a–), Le(a+b–), Fy(a+b+), Jk(a+b–), Xg(a+), Do(a)+, Co(a+b–), Vel+, I+. (2) B, MsNs, P_2, Tj(a+), CDe/cde, Lu(a–b+), kk Kp(a–b+) Js(a–), Le(a+b–), Fy(a+b–), Jk(a+b–), Xg(a+), Do(a+), Co (a+b–), Vel+, I+. Ashby counts [12] revealed 45% A_1B D-negative cells and 55% B D-positive cells, supporting the original 50:50 estimate.

Lewis antigens and secretions. Figure 1 shows that both the separated red cell populations were Le(a+b–). As Mrs. T. R. secreted B and H substances in her saliva, RACE and SANGER [15] pointed out that in these respects she is very similar to the Detroit example of dispermy [1, 16] who secreted A and H substances although all his red cells were Le(a+b–). BEATTIE *et al.* [1] deduced that the tissue formed from the minor (10%) genetic product at fertilization in their case possessed the genes

sese Lele and was capable of forming enough Le^a substance to coat the red cells of both populations. Since Mrs. T. R.'s father was dead and her mother was not tested for secretion it was impossible to decide whether the tissue producing her group B D-positive cells was genotypically Sese Lele and her Lewis groups really Le(a–b+), as suggested by BEATTIE *et al.* [1] for the major (90%) A_1O component in the Detroit example, or was Sese lele with cells Le(a–b–).

Two of Mrs. T. R.'s children were group O (fig. 1), strongly supporting the view that her reproductive cells and the tissue that was producing her group B, D-positive red cells were derived from the same original component at fertilization. If her A_1B, D-negative red cell-producing tissue had been associated, she would have had only A or B children. It was unfortunately impossible to assess with certainty the maternal and paternal Lewis and secretor gene contribution in this case, but RACE and SANGER [15] suggested that Mrs. T. R.'s mother was very probably B Sese, and her father had contributed A_1, se and O, Se sperm.

Leucocytes. HL-A grouping showed that there was an excess number of HL-A antigens present in Mrs. T. R.'s peripheral circulation. This is consistent with the existence of 2 different leucocyte populations. The antigens found were at the first (LA) locus: HL-A1, HL-A10 and W28 and at the second (Four) locus: HL-A8, HL-A13 (doubtful) and W5.

In all the reactions that were considered positive only half the number of leucocytes present reacted with each antiserum. This was in accordance with the 50:50 distribution estimated for the 2 populations of red cells. The Turin example of dispermy [4, 10], in which the lymphocytes were reported to differ by 2 HL-A haplotypes, was said to be genotype ac and bd in approximately equal proportions.

Skin mosaicism. The skin of both of Mrs. T. R.'s thighs was completely covered with fine, intimately interwoven, striations of light and dark-brown pigmentation. The skin of her trunk was said to be marked in the same manner, but only the lighter of the 2 shades of pigmentation was observed on the head, arms and shoulders. This type of skin mosaicism is quite unlike the case reported by ZUELZER *et al.* [1, 16] or that of COREY *et al.* [5, 11] in which skin 'patchiness' was described. The former (the Detroit example) had multiple small discrete areas of darker skin on the face, with fairly symmetrical wedges and bands extending in a lateral direction from both the anterior and posterior midline on the trunk, buttocks and thighs. The latter (the Vancouver example) had large mottled areas, most conspicuous on the abdomen where they formed patches of

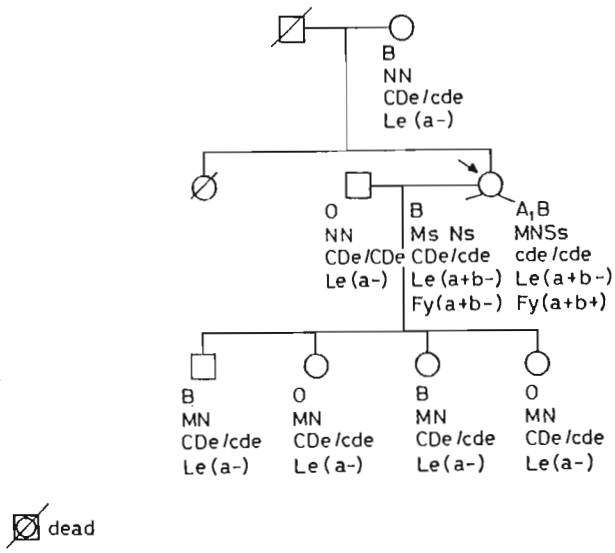

Fig. 1. The relevant blood groups of Mrs. T. R. and her family. Note: the Fy groups were determined subsequent to the family study.

light and dark pigmentation bordering alternately on the midventral and lateral lines; the upper left and lower right quadrants being darker than the alternate areas. No biopsy was made of Mrs. T. R.'s skin tissue.

Karyotype. 16 karyotypes prepared from cultured lymphocytes from Mrs. T. R.'s peripheral blood were normal (46,XX).

Discussion

Previous authors [1, 3–11, 14–16] have assumed that double parental contributions are responsible for the physical and serological anomalies observed in cases of dispermy. Mrs. T. R.'s normal XX karyotype and the fact that she has borne 4 healthy children clearly indicates that if her father contributed 2 sperm at her conception, both carried an X chromosome. She is the first example of dispermy in which the chromosome complement is XX/XX; for in 6 of the earlier cases XX/XY karyotypes were demonstrated, and the seventh case was a mongol child with normal male sex chromosomes.

The approximately 50:50 distribution of Mrs. T. R.'s double population of red cells and leucocytes more closely resembles the Seattle, Van-

couver and Turin examples [4, 5, 8–11] of dispermy than those of Detroit, Wisconsin, Oslo and Glasgow [1, 3, 7, 14, 16] where 90:10, 85:15, 80:20 and 97:3% distributions were demonstrated, respectively [15].

The absence of visible skin mosaicism in the upper-most part of Mrs. T. R.'s body may indicate that only one of her lines of cellular tissue predominated in this area.

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Request reprints from: Miss P. MOORES, B. Sc., Scientific Officer, Natal Blood Transfusion Service, 149, Prince Street, *Durban* (South Africa)

Distinguishing between twin and dispermic chimerism

The diagnosis of twin chimerism is usually easy: the existence of a fellow twin who also has mixed blood settles it, and even the history of a fellow twin is helpful. When there is no twin or history of one the decision can be difficult for there remains the theoretical possibility of a resorbed twin or perhaps even of a graft *in utero* from the mother.

Dispermic chimerism would be established if fibroblast culture showed chromosomal mosaicism or if the skin were patchy in colour. If a chimera had an XX/XY karyotype he would probably not be fertile if he were of the dispermic type and he might be expected to have some abnormality of his external genitalia. An exception is the Detroit propositus (Table 93) who now has three children.

We have placed the chimera studied by Battey *et al.*[98] (No. 20) at the bottom of Table 92. She was outstanding in having no history of being a twin, nor was there any record of abnormal tissue born with her. Nevertheless, she is here classified as a twin chimera because, though her lymphocytes are XY/XX, she is fertile; furthermore her fibroblast cultures were all XX and her buccal smear female. The theoretical possibility of a graft *in utero* from her mother is ruled out by her XY cells.

We presume we are right in listing as twin chimeras the three propositi whose twins are dead (Table 92). However, a lesson in jumping to conclusions is neatly given by the Durban II dispermic chimera (Table 93). This baby girl might have been listed as a twin chimera had not Moores shown that the male twin did not have the same blood groups as either cell line of the propositus, whose lymphocytes, incidentally, were subsequently found to be all XX.

Table 93. Dispermic chimeras

References	Propositus and relatives	Characters demonstrating mosaicism in propositus: Markers	Lymphocytes	Others	Double maternal contribution
DURBAN I					
Moores[99], 1966	Mrs T.R., aged 27	50% *BO, Ms/Ns, R¹r*, Sec	all XX	extensive mottling of skin (Mrs T.R. is a Tamil.)	
		50% *A₁B, MN.S, rr, se se*			
	father	dead			
	mother	*B, NN, R¹r Se se*			
	husband	*OO, NN, R¹R¹*			
	children	two *BO, MN, R¹r*			
		one *OO, MN, R¹r*			
		one *OO, NN, R¹r*			
DURBAN II					
Moores[109], 1969	Negro, female aged 6 weeks	*BO, MS/NS, Fy^aFy^b*	all XX		MNSs
		OO, Ms/Ns, Fy^bFy^b			
	mother	*A₂O, NS/Ns, Fy^aFy^b*			
	male twin	*A₂O, MNSs, Fy^aFy^b*			

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Zulu XX/XX Dispermic Chimaera from Natal with Two Populations of Red Blood Cells and Patchy Skin Pigmentation

Phyllis P. Moores[a], *Winifred M. Watkins*[b], *Pamela Greenwell*[b], *D. Dunn*[c], *Angela Bird*[a]

[a]Natal Blood Transfusion Service, Durban, Natal, South Africa; [b]Division of Immunochemical Genetics, MRC Clinical Research Centre, Harrow, Middlesex, UK; [c]Department of Human Genetics, South African Institute for Medical Research, Johannesburg, Transvaal, South Africa

Abstract. The chimaera is female and has two children. Her blood contains 99% group 0, type AcP:BA, Pep-A:8-2 and 1% group A_2B, type AcP:RA, Pep-A:1 red cells. *H*-, *A*- and *B*-transferase activities were demonstrated in her serum. The level of the *H* enzyme activity is low but is at the lower end of the normal range for group 0 persons. The levels of the *A* and *B* enzymes are also low but are higher than expected in a person with 1% A_2B red cells in the blood. The levels of the *A* and *B* enzymes indicate that tissues other than the chimaera's haemopoietic tissue carry her genetically A^2B cell line and are contributing the corresponding transferases to her plasma. Gross patchy skin pigmentation is present on the upper part of her body. The chimaera has evidently inherited two dissimilar germ nuclei from each parent.

Introduction

In human dispermic chimaeras the tissues are composed of cells derived from two genetically different cell lines [1]. For unknown reasons the zygotes or early cell morulas of dissimilar twins developing in utero are believed to merge and become one individual. The separate genetic origins of their cell lines distinguish chimaeras from mosaics who characteristically originate from a single zygote [2]. The literature contains counts of XX/XY hermaphrodite but few of XX/XX female dispermic chimaeras [1]. The first 2 females with double populations of red cells recorded were both from Natal [1, 3–5]. Mayr et al. [6] reported the 3rd female but two populations of red cells were not observed in her blood.

This paper presents details of the Zulu woman who is the 4th XX/XX female dispermic chimaera to be reported and the 3rd with a double population of red cells to be found in Natal.

Case Report

The chimaera, O.M., was identified when she gave a blood donation. During the preliminary ABO grouping, her red cells were typed as 0 but anti-A,B antibodies were not detected in her serum. Her blood was therefore referred for further studies. These showed that she has two populations of red cells and that her serum contains anti-A_1 antibodies. A possible A_3B_3 or A_mB_m phenotype [7] or dispermic chimaerism was suspected when enquiries revealed that O.M. had not had a tissue transplant or recent blood transfusion and no evidence of a twin or of unexplained extra tissue had been observed at her birth. The most likely explanation was dispermic chimaerism for O.M. also has gross patchy skin pigmentation of the thorax [fig. 1 and ref. 4, 5, 8].

Results

On being tested with anti-A, anti-A,B and anti-B, the red cells of O.M. were agglutinated in mixed-field patterns. The agglutinates were very small and required moderately prolonged microscopic observation to detect them. All three antibodies were recovered readily from her cells in absorption-elution studies by the 56 °C heat technique. Polyagglutination was excluded by the negative reactions of her cells with *Arachis hypogea*, *Salvia sclarea*, *Dolichos biflorus* and *Glycine soja* lectins and with ABO-compatible human sera [9]. The H and I antigens of O.M.'s cells are of normal strength. Saliva inhibition tests showed that she is a non-secretor of ABH and Lewis substances. A triple mixture of A, B and 0 red cells in her blood was excluded when no further agglutination was

observed after 1 vol of her washed packed cells had been mixed with anti-A and another with anti-B, rewashed and retested – the first with anti-B and the second with anti-A.

In the family study (table I), no illegitimacy was observed. *Cis* A^3B^3 and *cis* A^mB^m genes were excluded in I-1 and I-2 as the ABH substances secreted and the antibodies in their sera are in accordance with their ABO groups. A^3 or A^m genes masked in I-1 and B^3 or B^m genes masked in I-2 were also excluded as they were not detected in II-1, II-3 or II-6. The groups of III-1 and III-2 are not informative. The genotype of I-1 is therefore almost certainly A^2/O and of I-2 B/O and it is clear that these two persons may have a child who has both A_2B and O red cells in the blood. The chimaera (II-4) is believed to have originated when two zygotes or post-zygote cell morulas, one containing maternal A^2 and paternal B genes and the other maternal O and paternal O genes, merged. For O.M. to be a normal female, both of her father's sperms must have carried X chromosomes. The ratio of the two populations of red cells in O.M.'s blood was estimated by comparing the number and size of the agglutinates detected using anti-A or anti-B with those observed when samples containing different proportions of A_2B and O cells were tested similarly. The findings showed that her blood contained approximately 1% A_2B and 99% O red cells.

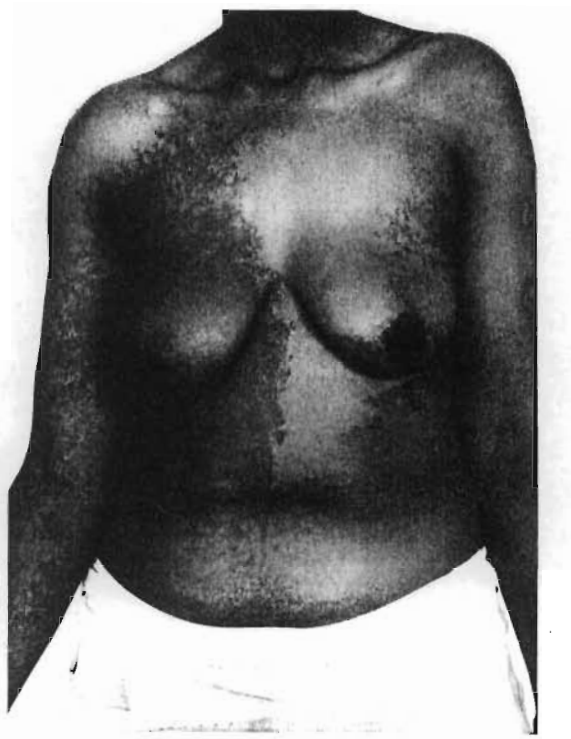

Fig. 1. The chimaera, showing the distribution of the pigmentation patches on her chest.

Table I. Data on O. M.'s family

Family No.	Sex	ABO	Serum contains	Saliva contains	MNSs	Lewis		Js		Xg[a]	RBC enzymes				HLA					
						a	b	a	b		GLOI	AcP	Pep-A		A	C	B	Bf	DR	DQw1
I–1	F	A_2	anti-B	A,H	MNs	–	–	+	+	+	2–2	RB	2–1	(a)	24	–	7	S	2	1
														(b)	w34	–	39	F	5	1
I–2	M	B	anti-A	B,H	MNs	–	+	–	+	–	2–1	AB	1–1	(c)	2	–	w58	F	5	3
													(8–1)	(d)	3	–	8	S	3	–
II–1	M	B	anti-A	B,H	Ns	–	–	+	+	+										
II–2	F	B	anti-A	B,H	Ns	–	–	–	+	+										
II–3	M	B	anti-A	B,H	Ns	–	+	+	+	–										
		A_2B									2–1	AR	1–1	(b)	w34	–	39	F	5	1
II–4	F		anti-A_1	non-sec	Ns	–	–	–	+	–										
		O									2–1	AB	8–2	(d)	3	–	8	S	3	–
II–5	M	A_2B	anti-A_1		MNs	–	+	+	+	+										
II–6	F	A_2	anti-B		MNs	–	+	+	+	–										
III–1	M	B	anti-A		MNs	–	–	–	+	–										
III–2	M	A_2	anti-B		MNs	–	–	+	+	–										

II–4 is O.M., the chimaera, with A_2B and O red cells in her blood. II–5 is O.M.'s husband and the father of III–1 and III–2. All generation II people are siblings of II–4. Generation I people are the parents of generation II. All family member's red cells typed as cDe (R_O), P_1, Lu(a–), K–, Kp(a–), Fy(a–b–), He–, I+.

Table II. Level of activity of *H*-, *A*- and *B*- transferases in the serum of family members and the control

Family pedigree number	ABO group	Incorporation of ^{14}C%			
		H	A		B
			pH 6	pH 8	
I–1	A_2	10.2	36.4	51.0	0
I–2	B	7.5	0	0	35.2
II–1	B	6.2	0	0	39.5
II–2	B	6.0	0	0	28.0
II–3	B	7.2	0	0	26.5
II–4	O/A_2B	4.7	1.4	3.0	3.9
II–6	A_2	13.4	21.8	44.0	0
Control	A_1B	9.5	55.0	32.2	26.0

II–4 is the chimaera.

Table III. *A* and *B* serum transferase activities in family members and the controls measured by conversion of O to A- or B- active cells

Source of serum transferase	Family pedigree number	ABO group	Anti-A titre	Anti-B titre
	I–1	A_2	2	0
	II–3	B	0	1,024
Chimaera	II–4	O/A_2B	0	128
Control		A_2B	16	1,024
Control		A_1	256	0

In the other informative blood group systems tested (table I), no evidence of a double population of red cells in O.M.'s blood was observed. However, analysis by starch gel electrophoresis of her separated cell populations and of her parents' cells in 11 different enzyme systems showed different phenotypes in the glyoxalase I (GLO I), acid phosphatase (AcP) and peptidase A (Pep-A) systems. The GLO I phenotypes were not informative. O.M.'s Pep-A:8-2 phenotype suggested a paternal exclusion but as the mobilities of the Pep-A 1 and Pep-A 8 isozymes are similar in the electrophoretic system used, we are unable to distinguish between the phenotypes Pep-A:1, Pep-A:8-1 and Pep-A:8. The phenotype of O.M.'s father is almost certainly Pep-A:8-1. Of the four HLA haplotypes in her parents, only two were identified in O.M.'s own leucocytes. Her leucocytes also produced only normal female (46,XX) karyotypes. No unusual G or C bands were seen. The same results were obtained with fibroblasts cultured from biopsies of the light and dark pigmented skin on O.M.'s back. Studies with fibrocyte lysates from the two skin biopsies showed that fibroblast differs from red cell AcP enzyme. The Pep-A enzyme failed to visualise and the G6PD enzyme results were not informative.

The levels of activity of *H*, *A* and *B* transferases in samples of serum from O.M. and members of her family were assayed by transfer of radioactively labelled sugars to the appropriate low-molecular-weight acceptors [10]. The results (table II) show that the level of the *H*-transferase in O.M.'s serum is lower than in the sera of her relatives tested and is similar to the levels in persons with A_3 and A_x cells [11]. The higher levels of the *H*-transferase in O.M.'s group A_2 than her group B relatives tested agrees with earlier observations on the levels of *H* enzyme activity known to be associated with the different ABO groups [11]. The level of the *A*-transferase activity in O.M.'s serum amounts to only about 6% of the activity in the serum of her mother, group A_2. The *A*-transferases in both O.M. and her mother's sera also exhibit higher activity at pH 8 than pH 6 which is characteristic of an A^2 gene product [12]. The *B*-transferase activity in O.M.'s serum is low and only about 10% of the level in the serum of her father, group B.

A second method used to study the *A*- and *B*-transferases in O.M.'s serum was by conversion of 0 into A- or B-active cells when incubated in the presence of the appropriate additives [10]. O.M.'s serum showed no capacity to confer A activity on 0 cells (table III), but this was expected as the transferases in A_2 persons normally have little activity when measured by this method. The serum of O.M.'s mother, group A_2, also gave only minimal conversion. The *A*-transferases in A_2B persons are known to have an increased capacity to convert 0 cells [13], but the activity is less than is expected when the transferase levels are assayed using low-molecular-weight acceptors. The *B*-transferase in O.M.'s serum converted 0 to B-active cells, but the degree of the conversion was only about one-tenth of that achieved using the serum of her brother, II-3, group B.

In the red cell stroma of O.M.'s father and three group B siblings (table IV), *B*-transferase activity was readily detected. Similarly, *A*-transferase activity was readily detected in the red cell stroma of her mother and sister, group A_2. The levels of the *A*- and *B*-transferase activities detected in her own red cell stroma were low, however, and the results are of doubtful significance. Nevertheless, from experience gained using artificial mixtures of red cells [14], and with prior knowledge of the transferase

Table IV. Level of activity of *H*-, *A*- and *B*- transferases in the red cells of family members and the control

Family pedigree number	ABO group	Incorporation of ^{14}C							
		H		A (pH 6)		A (pH 8)		B	
		cpm	%	cpm	%	cpm	%	cpm	%
I-1	A_2	322	0.4	3,798	1.1	9,114	2.7	0	
I-2	B	798	1.2	0		0		4,936	6.3
II-1	B	1,055	1.6	0		0		5,890	7.2
II-2	B	627	1.0	0		0		5,789	7.0
II-3	B	234	0.3	0		0		2,936	3.8
II-4	O/A_2B	682	0.9	110	0.1	139	0.2	155	0.2
II-6	A_2	531	0.8	624	0.9	1,618	1.8	107	0.1
Control	A_1B	488	0.7	2,614	2.7	2,026	2.1	2,735	3.3

II-4 is the chimaera.

levels in chimaeric twins [15, 16], we had not expected to detect *A* or *B* enzymes in a mixture containing only 1% of A_2B red cells.

Discussion

The normal female karyotype, feminine appearance and proven fertility of O.M. establishes that she is female. The A_2B, AcP:RA, Pep-A:1 and 0, AcP:BA, Pep-A:8-2 red cells in her blood and her patchy skin pigmentation favour dispermic chimaerism. The *H*, *A*- and *B*-transferases in her serum and sera of members of her family tested are those expected in relation to their serologically determined ABO groups. The levels of enzyme activity are also within the normal ranges for these enzymes. The levels of the *A*- and *B*-transferases detected in O.M.'s serum, however, are higher than expected if all the enzyme is being derived solely from her haemopoietic tissue; that is, if she is a genetically *00* twin whose 1% circulating A_2B cells originate from a graft in utero of primordial red cells from a group A_2B twin. Previous experience with twin chimaeras who have 1% cells of a certain ABO group in their blood has demonstrated that insufficient transferase is present for this enzyme to be detected by the methods currently available [16]. On the other hand, the level of *A* and *B* enzyme activity in O.M.'s serum is much lower than anticipated if she is a genetically A^2B twin chimaera whose 99% circulating 0 cells originate from a graft in utero of primordial red cells from a group 0 twin [15, 16]. These results therefore accord with the view that O.M. is a dispermic chimaera. They also show that the cell line carrying her A^2B genes is present in tissues other than her bone marrow. The low levels of enzyme activity detected indicate that her A_2B cell line constitutes only a portion of the tissues or organs which are contributing *A*- and *B*-transferases to her plasma. The level of *H*-transferase in O.M.'s serum is at the lower end of the normal range in 0 persons and is similar to the levels found in the sera of persons with weak A phenotypes [11]. The basis of the association between *H*-transferase level and ABO group is not yet understood.

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Dr. Phyllis P. Moores
Natal Blood Transfusion Service
PO Box 2356
Durban 4000 (South Africa)

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Pigment anomalies of the skin in the human chimaera: their relation to systematized naevi

G.H.FINDLAY AND P.P.MOORES

Department of Dermatology, University of Pretoria, and Red Cell Serology Reference Laboratory, Natal Blood Transfusion Service, Durban, South Africa

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SUMMARY

Double fertilization of the egg may lead to a chimaeric state, in which the cell composition of the individual derives from two zygotes. Some special types of naevoid hyperpigmentation may result Two new cases are added to the three published cases of chimaerism with skin manifestations.

Of all the reasons for a disturbance in skin colour, the rarest is perhaps that in which the individual is formed from more than one zygote. These are the true chimaeras, in whom differing sets of chromosomes are presumed to result from a dual fertilization, provided that other sources of cell mixture are unlikely. At the blastocyst stage in embryonic life, in the mouse at least, there are possibly only three cells which actually go to form the body of an embryo (Markert & Petters, 1978). If these three cells are derived from two different zygotes, the starting proportions of the mixture will obviously lie at two-thirds to one-third.

When two sperms fertilize an ovum which contains an extra nucleus (e.g. a polar body) a chimaeric state results. It will not be suspected if the embryonic body happens to stem exclusively from one of the two available cell lines. However, if the body should contain cells with differing sets of chromosomes, this mixture of cells may eventually be revealed through the individual possessing a mixture of two kinds of blood or by intersexuality, should there happen to be an XX/XY mosaic of the sex chromosomes. An XX/XX mosaic will not disturb sexuality. Such spontaneous cell mixtures in chimaeras may also cause the skin to manifest two shades of colour, though not necessarily so (Race & Sanger, 1975).

Even if the original cell mixture, existing in a state of mutual tolerance in the embryo, could have been expressed as a simple mathematical proportion, cellular movements, selection and change with space and time during development are bound to disturb the initial ratio (West & McLaren, 1976; Stephens, McIvor & Wagner, 1977; Falconer & Avery, 1978).

Known zygote mixtures also affect the phenotype in ways that may be attributed to the cells of neither parent. When a healthy embryo is fused with an abnormal or lethal one at an early stage, the cells of the poor lines may be 'rescued' by the normal ones (Bennett, 1978; Le Stevens, 1978).

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The effects of a cell mixture may be imitated in the female by partial or total inactivation of one or other X chromosome, when the phenotypes are conspicuously dissimilar. For instance, should one of these X chromosomes carry a translocated gene for albinism, patterns of pigmentation arise which look remarkably like certain human chimaera skin patterns. Thus, in the so-called Cattanach translocation in mice, a patchwork of alternating dark and light areas results. They meet sharply in the ventral midline, and are clearly mismatched with one another (Fig. 1F). Similarly in the brindled mouse, despite smaller colour differences in the transverse barring, there may also be a gross asymmetry in colour development on either side of the midline (Fig. 1E). These findings and their interpretation are accessible in a valuable paper by Grüneberg (1969).

Grüneberg's views are in remarkable harmony with Thom's (1975) later concept of morphogenetic stability, arising by 'catastrophe' from a foregoing unstable state. The stable picture comes about by means of a threshold phenomenon, based upon the group behaviour of cells. It is preceded by a phase of instability due to combined inhomogeneities between genes, chromosomes and cells in a tissue. It is a harder concept to grasp than the belief in a simple pathway to pattern production. The simple view suggests a straightforward onward movement from gene-to-cell-to-tissue-to-pattern. Although this simple pathway provisionally accounts for certain animal colour patterns, it suffers from being generally unworkable, as Grüneberg cogently argued, and as observers of other mammals have long had reason to suspect.

Human chimaeras with patchy skin colour are now five in number—three are described in existing publications, to which we add two. One of our cases was reported earlier (Moores, 1973) but her skin was not fully described at that time. This lack has now been remedied. The other chimaera, which was discovered later, has not been reported before.

SKIN FEATURES IN THE REPORTED CASES OF HUMAN CHIMAERAS

Zuelzer, Beattie & Reisman (1964)

An 18-year-old 'male' of mixed Negro, Caucasian and Amerindian descent showed topographical differences in skin pigmentation. He was described as having skin of a pale milky coffee colour, some 10% of which was darker in certain sharply outlined areas. The areas consisted of irregular pigmented patches on the face, and darker wedges and bands abutting on the anterior and posterior midlines of the trunk. Figures 1B and C show the pigmentation pattern as it appears in the published pictures.

A prospective blood donor, this individual was found to have both group A and group B cells, and a slight gynaecomastia which was the sole clinical sign of intersexuality. Fibroblast cultures from the normal pale skin areas yielded XY cells only. The dark skin gave rise to more slowly growing fibroblasts, yielding XY and XX metaphases in a roughly 10:1 ratio. Tetraploid cells were common throughout.

Corey et al. (1967)

A white 'male' child was born with a bifid scrotum, extreme hypospadias, one ovary and one testis, although male characteristics predominated. The skin showed some general mottling, but the anterior abdominal wall on either side of the midline exhibited a darkening in roughly reciprocal quadrants. These darker areas tanned readily (Fig. 1A). The fibroblasts of the skin in two diagonally placed pale areas yielded 94% XX and 6% XY cells, while the corresponding dark zones yielded 60% XX and 40% XY, and 68% XX and 32% XY cells respectively.

Fitzgerald, Donald & Kirk (1979)

A white 16-year-old 'male' had hypospadias, gynaecomastia, one ovary and one testis. There were

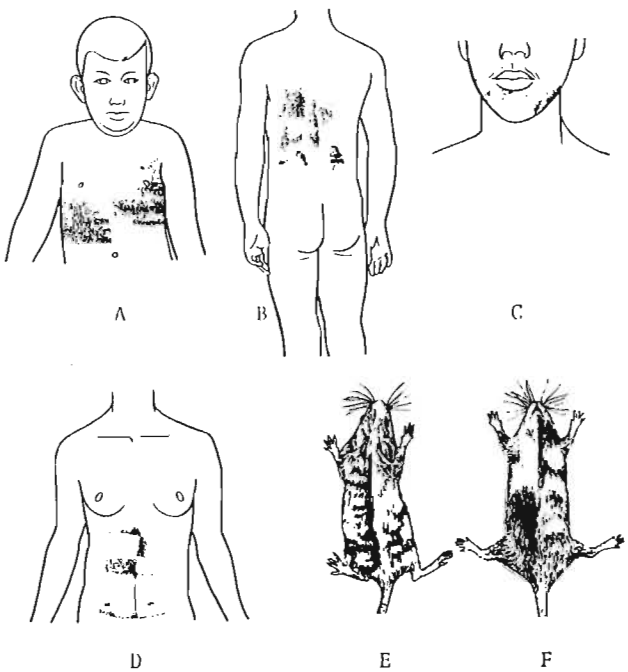

FIGURE 1. (A) Skin pigment mosaicism of the abdominal area. Redrawn from Corey *et al.* (1967). (B) Irregular localized pigmentation of the torso. Redrawn from Zuelzer *et al.* (1964). (C) Patchy pigmentation of the face. Redrawn from Zuelzer *et al.* (1964). (D) Segmental distribution of skin pigmentation of the trunk and gynaecomastia. Adapted and redrawn from Fitzgerald *et al.* (1979). The lower abdomen shows what we presume to be the two biopsy sites, taken from skin areas with relatively little colour contrast. (E) Brindled ($Mo^{br}/+$) female mouse aged 47 days, with dorsal midline effect. Dark and light areas meet sharply along the midline. Redrawn from Grüneberg (1969) (F) Flecked female mouse 61 days old with Cattanach's translocation. Crossed midline effects on the venter in the absence of similar dorsal effects. Redrawn from Grüneberg (1969).

segmented areas of darkened skin on either side of the abdominal midline with rectangular flag-like outlines. The patches suggested a checkerboard (Fig. 1D). Some horizontal dark lines and a marked linea nigra were also present. Fibroblasts from dark and light areas both yielded XX metaphases only, but a laparotomy incision for pelvic exploration yielded XX and XY fibroblasts in a 2:1 ratio. One presumes that the laparotomy sample was obtained in the midline from the region of the dark linea nigra.

CASES OF CHIMAERISM FROM NATAL

In more than a million routine blood tests at the Natal Blood Transfusion Service, only two samples were encountered suggesting a true chimaerism. Both were from 46 XX chimaeras, and both showed some remarkable skin features.

Case 1 (Moores, 1973)

This Indian woman was re-examined in 1979 to review the skin anomalies which were merely mentioned in the publication of 1973. On this more recent occasion the earlier blood findings were

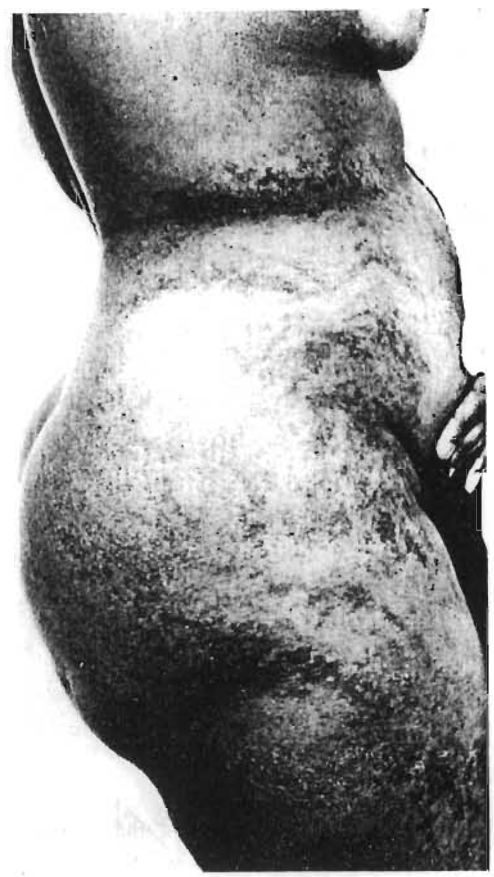

FIGURE 2. Case 1. Chimaera. Mottled, reticulate and striate pigmentation.

also confirmed. They are shown, together with new red cell enzyme results in Table 1. For other details, the original publication should be consulted.

In her case, widespread areas of pigment increase occurred all over the skin, arranged principally in striae. The areas were much more marked on the right half of the body, and were intensified where the striae ran together on the forearm or lower abdomen, and at sites of friction or sun exposure. These darker areas could be analysed into four interrelated patterns or grades of pigmentation, the mildest alteration being (i) a fine speckling or mottling, blending into (ii) a coarser reticulation consolidating into (iii) fine streaks or striae thickening into bands, and by fusion of bands into (iv) block-like dark masses.

The pigmented striae were to be found at or near the midline, down the length of the limbs and encircling the trunk. Their manner of arrangement recalled the pattern of epidermal naevi disposed along the so-called naevus lines of Blaschko. A number of these features are illustrated in Figs 2–4.

No histological examination was made.

Case 2

A healthy Black Zulu woman aged 30 donated a unit of her blood to the Natal Blood Transfusion Service. Though apparently of group O, her serum contained a weak anti-A_1 allo-antibody only.

TABLE 1. Red cell groups, red cell enzymes, leukocyte groups, and fibroblast cultures in two chimaeras from Natal

	Case 1		Case 2	
Red cell groups	55%	45%	99%	1%
ABO	B	A_1B	O	A_2B
MNSs	ss	Ss		
Rhesus	Rh_1rh	rh	No other differences were detected probably because of the small size of the minor cell population and the remarkable similarity of the parent's blood types	
Duffy	Fy (a+b−)	Fy (a+b+)		
Red cell enzymes				
Acid phosphatase (AP)	AB	A	AB	AR
Glutamyl pyruvate transaminase (GPT)	2	2–1		
Adenylate Kinase (AK)	1	2–1		
Esterase D (EsD)	2	2–1		
Peptidase A			8–2	1–1
Leukocyte Groups				
1st locus (LA)	HL-A1, W28	HL-A10		
2nd locus (Four)	HL-A8, W5	HL-A13 ±		
Fibroblast cultures	Not performed		No cell or chromosome abnormalities inside or outside the pigmented zones	
Chromosomes	All normal, 46XX		All normal, 46XX	

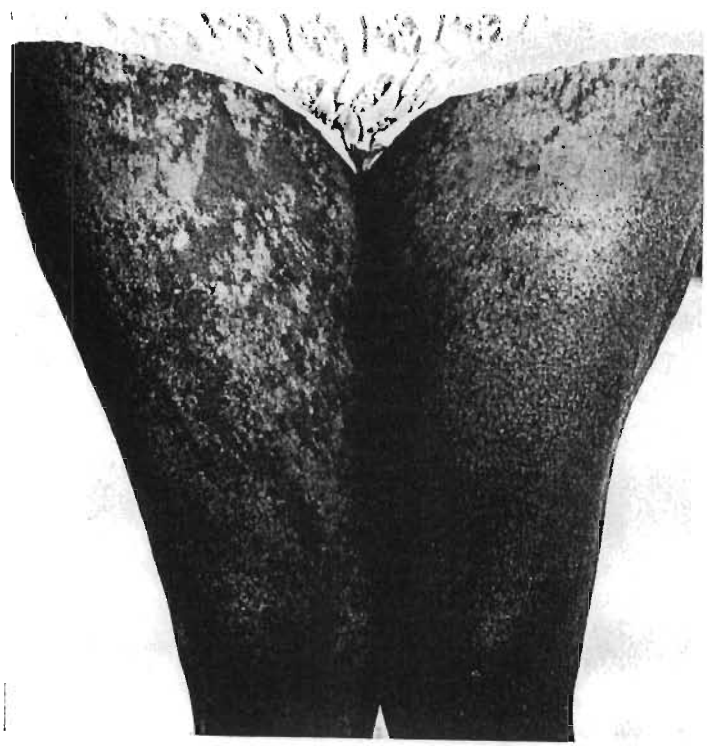

FIGURE 3. Case 1. Chimaera. Expansion of pigment network and bands into darker, block-like masses and wedges.

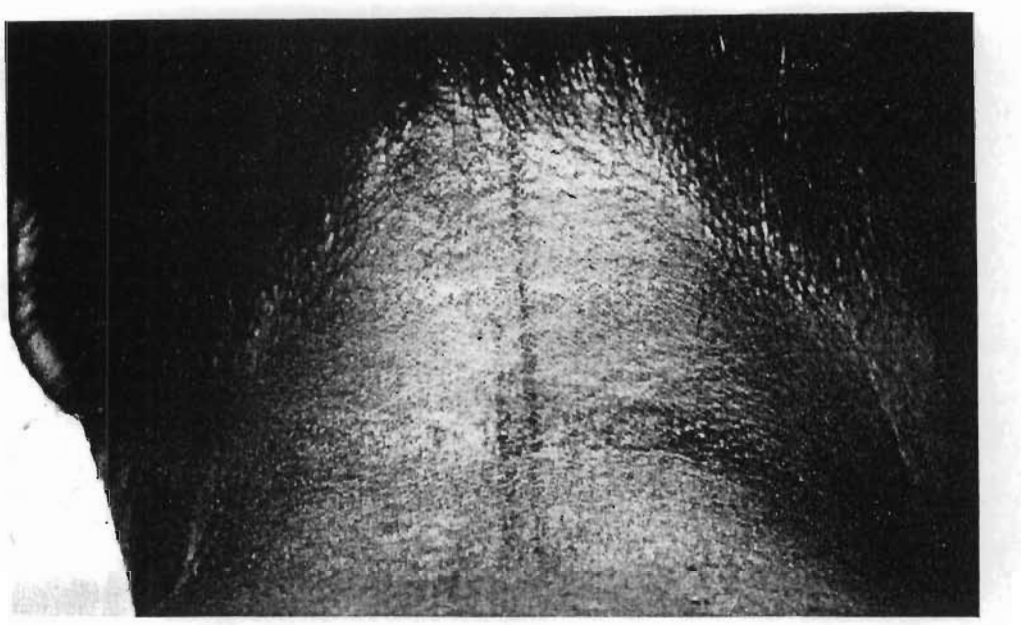

FIGURE 4. Case 1. Chimaera. Vertical arranged parallel pigment lines in nape of the neck. Frictional pigmentation, horizontally disposed from a necklace, more prominent on the right than left.

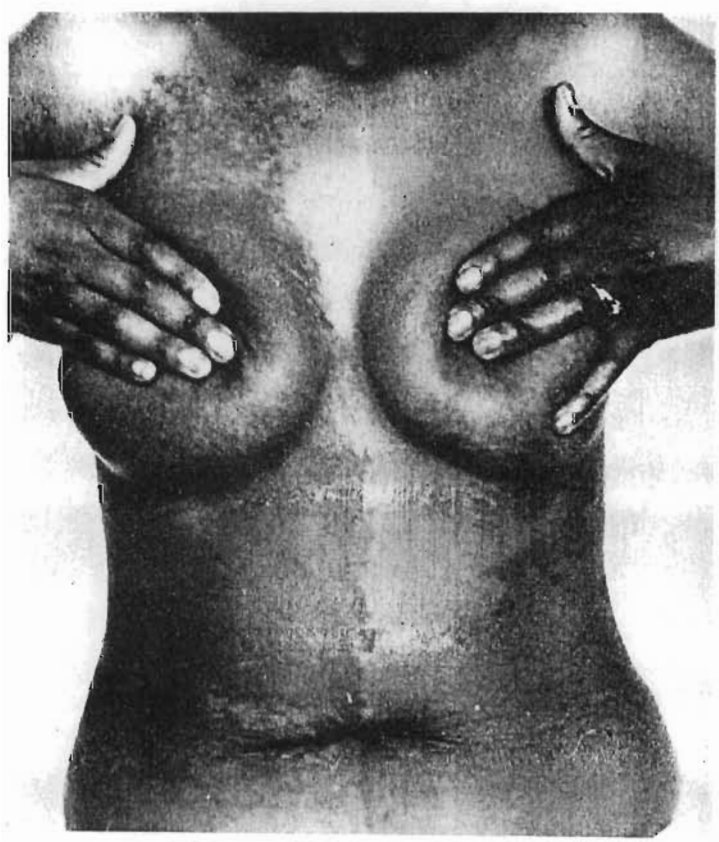

FIGURE 5. Case 2. Chimaera. Increased pigmentation on the right is confined between the midline, the 'centaur' line at the waist below, and the first intercostal space above. On the left a few bands of pigment reach the lateral edge of the rectus sheath.

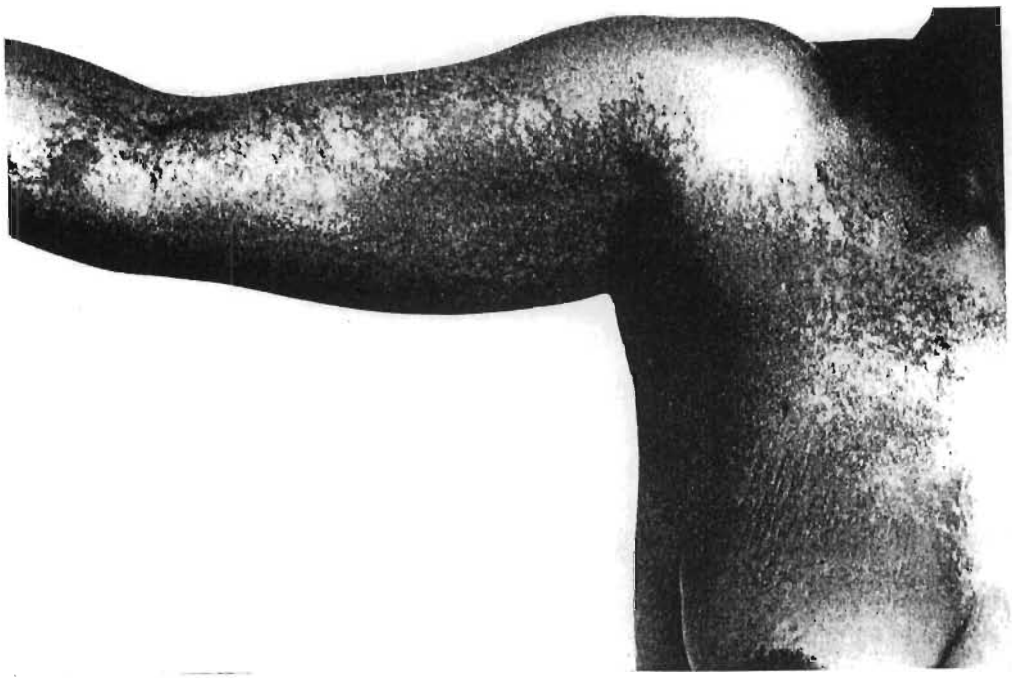

FIGURE 6. Case 2. Chimaera. Spray-like and granular pigmentation forming café-au-lait like patches, e.g. just below the elbow. Posterior half of the arm circumference mainly affected, medial to the area where Voigt's line would lie.

Further studies showed that her blood contained 99% group O and 1% A_2B red cells. These and other findings are listed in Table 1.

She was married, with two normal male children. Her pale brown skin was marked by large patches of unusually dark brown pigment, which had evidently not changed since birth.

The patches of abnormally dark skin colour (Figs 5–7) were restricted to the trunk and backs of the arms. The upper and lower limits of this zone could be clearly marked out by horizontal lines. By abducting the arm to a right angle, the upper limit was seen to run horizontally from the 2nd thoracic spine behind and the 2nd rib in front out to the head of the ulna. The lower limit ran horizontally round the trunk at the level of the anterior superior iliac spine. This pigmented zone abutted roughly at right angles on the dorsal and ventral midlines, tending to make square-cut edges. The quota of pigmentation between these limits was very different on the two sides of the body. On the right side the whole region was packed with heavily pigmented areas of skin, while the left side showed only a mild and incomplete suggestion of the full picture.

In contrast to our previous case, this subject's pigmentation, on the right side at least, could be graded into (i) a paler, fine, spray-like pigmentation, with a fine granularity of 1 mm or less, present at the upper limit of the area. Further down, the area contained (ii) larger solid rounded patches of varying darkness, resembling the café-au-lait spots in neurofibromatosis, with a paler background interlaced between them. At the lower limit of the area the pigment was (iii) confluent and darker than elsewhere. On the left side the pigmentation did not reach the midline, either in front or behind, and was arranged anteriorly in solid fingers pointing towards the rectus sheath and posteriorly in finer mist-like patches extending over the left shoulderblade. Skin from pale and dark areas of the

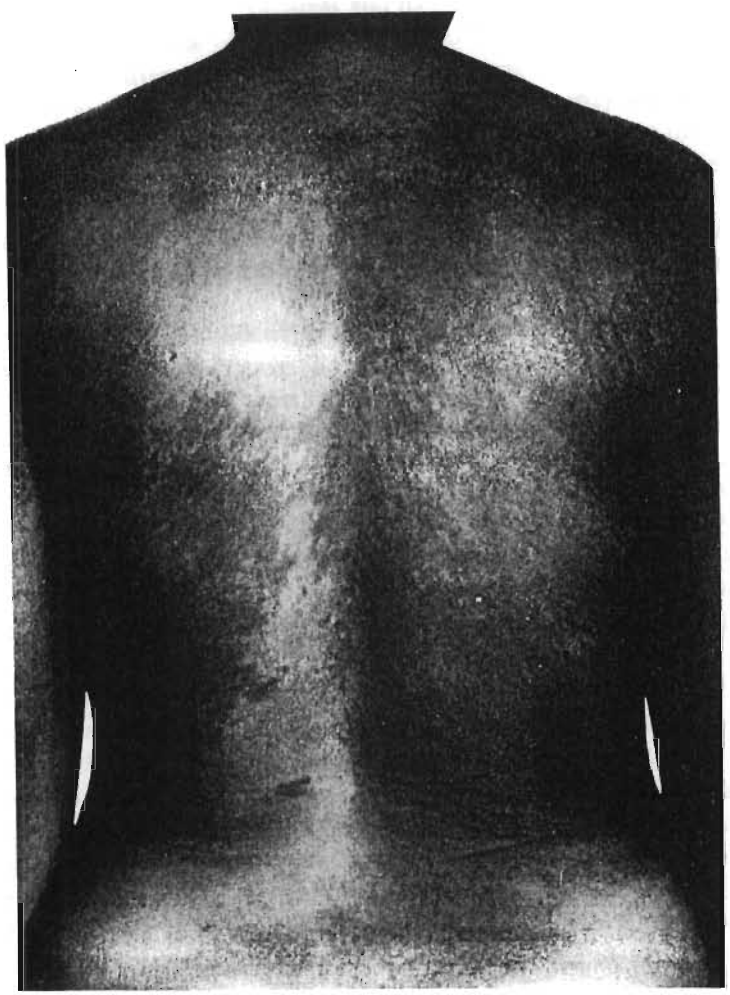

FIGURE 7. Case 2. Chimaera. Pigment limits on the right side of the back are seen at the level of T_2, the midline and horizontally in the lumbar region. On the left the pigment is finer and is mostly situated well away from the midline.

upper back were compared histologically with H & E and silver stains. Both were normal in structure and cell composition, but possibly in the skin from the darker area the melanocytes were plumper, producing more pigment, loading the basal and suprabasal cells of the epidermis more heavily. Nevertheless, epidermal pigmentation was uneven in both specimens.

Under the electron microscope some slight differences were seen between the epidermal cells of the two pieces of skin. Looser textured nucleoli and smaller, conglomerate melanosomes were seen in the darker skin, though perhaps these lay within the range of normal.

DISCUSSION

It is impossible to guess what a human chimaera ought to look like, and so our two chimaeras naturally surprised us in several ways. Their skin pigmentation could be divided into two regions: (i) a major region normal in appearance and (ii) a minor region, darker than one might expect if the skin had

belonged to 'the other twin'. Evidence published by others also suggests that the fibroblasts in chimaeras may be more mixed in the darker skin areas and more homogeneous for one or other of the two cell lines in the normal skin areas.

Three main types of pigment pattern are recognizable in chimaeras. (1) A flag-like rectangular pattern. This is seen also in generalised naevus spilus (Davis & Shaw, 1964) and the Cattanach translocation (Grüneberg, 1969). (2) A pattern of rounded units. This is analogous to the café-au-lait patches in Von Recklinghausen's disease. (3) A striate pattern. This imitates the systematized epidermal naevus.

The increased pigment is strikingly collected into large compartments often with straight edges and sometimes having a box-like shape. Checkerboard patterns and marked asymmetry are evident, almost indicating that increased pigment at one block-like site may actively prevent its occurrence in the opposite half of the body across the midline, or up and down on the same side.

It is not really clear why the darker skin regions in chimaeras have such clean and strict outlines, or how they interact with the paler reciprocal areas in and around them. Even the innocent-seeming dorsal and ventral midlines, the obscurer naevus lines of Blaschko or the more common pigment demarcation lines (Matzumoto, 1913; Selmanowitz & Krivo, 1975) in normal individuals have not been accounted for, although they often serve as barriers for normal and pathological processes, as they do in chimaeras as well. Another line which may usefully be added to this assembly of unexplained lines lies horizontally across the lower abdomen and was evidently a pigment barrier in our second chimaera. Interestingly, this line is also the cut-off point in cases of superior lipodystrophy. We have called it the 'centaur line'. In this position the human part of a centaur changes into an animal, just below the waist, according to mythical tradition.

It would be attractive if one could discover a unitary scheme to account for chimaeric, naevoid and genetic patterns among abnormalities in the skin. The rival possibilities of a stem cell, organ system, tissue layer or cell mixture have to be considered, and these need to blend satisfactorily with induction processes, threshold phenomena and action at a distance. Judging by the chimaeric state, it seems that the somatic mesoderm and the neural crest contributions to the connective tissue appear to have as good a claim as any to cause the stepwise demands for pigment induced by this cell mixture on the epidermis. In the complex situations we meet with in clinical dermatology, a basic scheme of melanoblast clone migration (Mintz, 1974) is less satisfactory than one in which the melanoblast is merely the servant of a far more elaborate process, the colour level then being induced in the epidermis by the composition of underlying connective tissue.

ACKNOWLEDGMENTS

The authors thank Professor Trefor Jenkins for the red cell enzyme studies and the skin tissue cultures, Professor Peter Brain for the photographs of the chimaeras, Professor Ian Simson for the histology and electron microscopy, Mrs Angela Bird for referring Case 2, the two chimaeras and their families for numerous blood samples and willing co-operation, and the South African Medical Research Council for support. David Dunn performed additional red cell enzyme studies.

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CHAPTER IX

POLYAGGLUTINABLE RED CELLS

IX.1 PAPERS

Severe haemolytic anaemia in an adult associated with anti-T
Paper 29 by Moores, Pudifin and Patel

Haemoglobin M-Hyde Park associated with polyagglutinable red blood cells in a South African family
Paper 44 by Bird, Kent, Moores and Elliott

Enhanced reaction with *Vicia graminea* lectin and exposed terminal N-acetyl-D-glucosaminyl residues on a sample of human red cells with Hb M-Hyde Park
Paper 46 by King, Liew, Moores and Bird

Lectins from *Griffonia simplicifolia* (GS II) and *Vicia hyrcanica* interact with terminal N-acetyl-D-glucosaminyl residues on a sample of human erythrocytes with Hb M-Hyde Park
Paper 45 by King, Liew, Moores and Bird

IX.1 INTRODUCTION

Red cells become polyagglutinable when their membranes are altered in some way. The alteration may be either transient, due to the enzymes produced by certain bacteria or viruses in infections, or permanent, due to somatic or inherited mutations. Normally hidden antigens (crypt or latent or the cells released earlier than usual from the erythropoeitic tissue) are exposed. Occasionally, no membrane change occurs, but the red cells are agglutinated by polyagglutinins in normal human sera which do not affect normal red cells. Polyagglutination was described as early as in 1925 by Huebner [Issitt, 1985, p458]. By 1930, both Thomsen and Friedenreich had made substantial contributions [Friedenreich, 1930, p128], and it subsequently became known as the Huebner-Thomsen-Friedenreich phenomenon. The current types of polyagglutination are T, Tn, Tk, Th, Tx, VA, Cad, acquired B, HEMPAS and NOR.

In polyagglutination due to T-sensitization, the red cells lose their M and N antigens, are agglutinated by the anti-T normally present in adult serum and by *Arachis hypogea* (peanut) anti-T

lectin and are not aggregated by polybrene or by protamine sulphate [Issitt, 1985, p458-462]. The plasma of infants of from birth to about one year of age does not normally contain anti-T. In two reported cases of anaemia and haemolysis in infants, the cause was traced to anti-T in the plasma of the adult blood transfused. Anti-T is not usually identified in the plasma of adults whose own red cells are T-sensitized. The explanation may be that all their anti-T has been adsorbed by their own red cells. Alternatively, they may have immune tolerance or immune paralysis.

IX.2 COMMENTARY

IX.2.1 Attainments in Durban, 1961 to 1991

IX.2.1.1 T-sensitization

In Paper 29, the authors reported the case of a Black woman with a stab wound in her back whose red cells had agglutinated spontaneously in samples of her blood drawn during a sudden haemolytic crisis. Anti-T was identified both in her serum (titre 256 at 4°C, 128 at 10°C, 64 at 22°C and 0 at 37°C) and in red cell eluates. The woman's red cells were strongly agglutinated by anti-T lectin, were agglutinated by some but not all anti-M and anti-N reagents and were mildly aggregated by protamine sulphate. Their H antigen was not increased in strength, compared with that of normal group O red cells, and they gave negative results with incomplete anti-c, anti-e and anti-D reagents used by saline technique. The findings indicated that the woman's red cells were T-sensitized, but they also suggested that the process of T-activation was incomplete. Possibly, her plasma did not contain normal enzyme-inhibiting substances, or the substances had been rendered temporarily inactive. Auto-immune haemolytic anaemia was diagnosed. As no bacteria were isolated (a blood culture was not done), the identity of the organism producing the neuraminidase that had caused the T-activation was not discovered. Pulmonary infection was nevertheless suspected. The woman's condition responded rapidly to treatment with antibiotics, and soon she was able to leave hospital. The method of red cell destruction (haemolysis) in her was

difficult to explain. Anti-T was not an IgG antibody and did not normally fix complement. Their elimination may have been the result of physical damage caused by the removal of some of their N-acetyl-neuraminic acid. Consequently, their agglutination by anti-T may have been merely a coincidence; otherwise, although well-developed, the woman's anti-T may have been only loosly attached to her red cells.

IX.2.1.2 Polyagglutinable red cells and Hb M - Hyde Park

The discovery of "black-coloured" blood in a Coloured blood donor [paper 44] led to the identification of an entirely new type of polyagglutination. The donor's family was the first to be recorded in which close association between a red cell phenotype and a haemoglobin variant was clearly demonstrated. The donor was found when his red cells unexpectedly gave weak positive results in several cross-matching tests. Initially, the mixed-field appearance of the results suggested that his group was not O but A_{bantu}. In the subsequent studies, however, his red cells were seen to be polyagglutinable. A family study was made, and "black-coloured", polyagglutinable blood was identified in 11 of the 35 members tested. The characteristics of the polyagglutination were: (1) the red cells were weakly agglutinated by many ABO compatible sera from adults; (2) the red cells were not agglutinated by *Arachis hypogea*, *Dolichos biflorus* or *Salvia sclarea* but were weakly agglutinated by *Salvia horminum* and BSII (GSII) and strongly agglutinated by *Glycine soja* and *Sophora japonica* lectins; (3) BSI (GSI) lectin agglutinated the red cells of the group A but not the group O family members; (4) *Vicia graminea* and other anti-N lectins and human anti-N but not monoclonal or rabbit anti-N agglutinated the group N and group MN family members' red cells more strongly than normal; (5) the anti-M reagents used reacted as expected with the group M and group MN family members' red cells; (6) the red cells gave stronger positive results than normal with anti-i reagents, and (7) the red cells gave negative results with anti-Dantu and incomplete Rh antibodies used in saline tests. The affected family members were all found to have rare haemoglobin M-type Hyde Park. This

haemoglobin is the result of a His to Tyr substitution in the peptide chain. The name suggested for the polyagglutination was "Hyde Park", but only if other persons with the same haemoglobin had similarly-affected red cells. The unusual haemoglobin and polyagglutination were unlikely to be the result of a single genetic change. Instead, the bonds which occur normally between haemoglobin and α-sialoglycoprotein molecules in aging red cells might have been affected more than usual by the precocious senescence. "Hyde Park" polyagglutination may have been the product of normal red cell denaturation occurring in unusual circumstances.

Since this case was published, Professor H. Franklin Bunn (Harvard, Boston, USA) has kindly given another explanation. He suggested that a mutation had occurred in the family in a gene for a red cell membrane protein situated close to the structural gene for ß-globin on chromosome 11. The polyagglutination and haemoglobin would therefore be entirely unrelated, other than by the proximity of the two genes.

Blood samples from a family member with "Hyde Park" polyagglutination and controls were tested in Oxford for their biochemical, immunochemical and other properties [paper 46]. The amount of sialic acid on the membranes of the family member's red cells was found to be approximately 90% of normal. The polyagglutination was not type Tn, for anti-T and anti-Tn were both present in the propositus' serum. Two unrelated membrane abnormalities were identified. In the first, the size of the sialoglycoprotein molecules varied widely and the O-linked oligosaccharide chains on these membrane components were mildly reduced. In the second, terminal N-acetylglucosamine was exposed on the membrane components of apparent molecular weights 88 000 to 130 000 and 46 000 to 73 000. These components probably corresponded to Band 3 and Band 4.5, respectively. As separate pathways existed for the synthesis of O- and N-linked oligosaccharides and the abnormalities were probably confined to the non-reducing terminal of these carbohydrate chains, the findings did not explain how a haemoglobin variant could influence both glycosylation

processes simultaneously. The heterogenous sialylation suggested that the red cells had not yet reached their adult form (as in other types of bone marrow stress caused by anaemia). Exposed terminal N-acetylglucosamine in a healthy individual in any case was unusual, and it indicated that the glycosylation was defective. The exposure was not due to depressed ß(1-4)galactosyltransferase, as large amounts of this enzyme were present in both the propositus' red cells and plasma.

The lectin, *Griffonia simplicifolia* or GSII, formerly known as BSII, is specific for N-acetyl-D-glucosamine. Both this and a new lectin, *Vicia hyrcanica* [Liew and Bird, 1987], were found to agglutinate polyagglutinable red cells with haemoglobin M-type Hyde Park [paper 45]. The weak positive results given by the red cells before enzyme-treatment implied that terminal N-acetylglucosamine residues were exposed on them *in vivo*.

Paper 29

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Severe Hemolytic Anemia in an Adult Associated with Anti-T

P. MOORES, D. PUDIFIN, AND P. L. PATEL

From the Natal Blood Transfusion Service, and the King Edward VIII Memorial Hospital, University of Natal, Durban, South Africa

An adult woman hospitalized because of a stab wound suffered a severe hemolytic crisis during which she was found to be profoundly anemic. There was evidence of increased red blood cell fragility and extravascular hemolysis. Her cells, which were T-activated, were agglutinated spontaneously *in vitro* by anti-T in her own plasma. They were also agglutinated by some, but not all, human anti-M and anti-N sera, and were mildly aggregated by protamine sulphate. A normal amount of H antigen was present and no agglutination occurred in saline by incomplete Rh antibodies. The findings indicate that T-activation was incomplete. It is suggested that the patient experienced a short episode of acquired autohemolytic anemia caused by the activity of her own anti-T during a period when her normal enzyme-inhibiting substances were either not present or had been rendered temporarily inactive.

RED BLOOD CELLS become polyagglutinable *in vitro* when their normally latent T antigen is exposed as the result of the removal of N-acetyl-neuraminic acid (sialic acid) from the red blood cell membrane by neuraminidase. Neuraminidase is an enzyme commonly produced by a variety of bacteria.[6,15,23,24] When the cells are altered in this way, they are said to be T-activated.[13] They then have no detectable M or N antigens, are not aggregated by Polybrene, and are agglutinated by anti-T, an antibody present in the serum of most human adults and many mammals, but not of infants. The type of agglutination seen is usually mixed fields of small agglutinates among many unagglutinated cells. Bird[3] found a convenient source of potent anti-T in saline extracts of peanuts (*Arachis hypogea*).

Polyagglutinable red blood cells *in vivo* have been reported on a number of occasions[8,14,25] usually accompanied by evidence of an infection, but very few of the affected persons were adults in whom the T-activated cells were associated with hemolysis or anemia.[11,26] Complications of this kind apparently occur more often in young children, especially in their first year of life.[22,26] Two cases have been reported in which infants suffered severe hemolysis during transfusion as a result of anti-T in the plasma of the donor blood.[21,29] Tn-activation is a related condition in which the affected cells are not agglutinated by anti-T or by Polybrene but are agglutinated by extracts of *Salvia sclarea* (clary)[9,10] and *Dolichos biflorus* seeds.[2,5,6,12,20,28] Tn-activation is more commonly associated with anemia in adults, but unlike T-activation the effect is not reproducible *in vitro*. Gunson, Stratton, and Mullard[17] questioned whether *in vivo* polyagglutination was caused by neuraminidase when they found that the serum of two patients with Tn-activated cells contained normal amounts of neuraminidase inhibitor substances. Tk-activation is a recently reported[7] intermediate type of polyagglutination in which the cells are strongly agglutinated by peanut extract, strongly aggregated by Polybrene, and their agglutination reaction is enhanced by prior treatment with papain. Tk-activation does not result in hemolysis *in vivo*.[7]

None of the previous reports has indicated that anti-T was present in the serum of the patients who had T-activated red blood cells while their disease was in progress. Gunson, *et al.*[17] explained this by presuming that anti-T was all absorbed onto the patient's own cells. Beck, *et al.*[1] proposed that the patient had developed a form of immune tolerance. Berman, *et al.*[2] concluded that immune paralysis had taken place. Bird[4] suggested

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that anti-T, which is really an isoagglutinin because the T antigen is normally hidden, should be considered a potential autoagglutinin. We observed that the cells of an adult woman agglutinated spontaneously in samples of her own blood taken during a hemolytic episode. Anti-T was found both in her serum and in eluates made from her cells which during this period were typically T-activated.

Case Report

The patient was a 38-year-old Zulu woman from a rural area of Natal. She had not had any blood transfusions but she had had several children. She had been in good health prior to being stabbed below the angle of the left scapula four days before coming to the hospital. On admission she complained of three days of severe chest pain, weakness, dizziness, and dyspnea. The patient was pale and febrile (temperature 39 C, pulse 130 per minute) and her liver and spleen were palpable. There were no signs of shock or jaundice, but some consolidation and evidence of a pleural effusion were detected at the base of her left lung. A blood count revealed a normocytic, normochromic anemia (Hb. 8 g/100 ml), with neutrophil leukocytosis (total white cell count 26,000 mm^3), some autoagglutination, and increased polychromasia in the blood film. There was an opacity in the lower half of the left lung, with no mediastinal displacement and no pneumothorax. Aspiration of the left pleural cavity was attempted. However, only 2 ml of clear yellow fluid was obtained, which was sterile.

Treatment commenced with 2 g ampicillin daily, and for the first seven days the patient's condition improved steadily. Her temperature returned to normal and the dyspnea subsided. She then suddenly began to deteriorate rapidly, with increasing pallor, weakness, and dyspnea. Her hemoglobin had fallen to 3.4 g/100 ml. Her white blood cell count was now 17,000/mm^3, reticulocyte count was 25 per cent, there was marked aniso- and poikilocytosis and increased polychromasia, and numerous normoblasts were in the blood film. Her red blood cell osmotic fragility was increased, serum glutamic oxalic transaminase rose to 55 units (normal 0 to 23 units), serum gamma globulin concentration was slightly increased, and serum bilirubin was 1.9 mgm/100 ml. The direct antiglobulin test appeared to be positive. Red blood cell polyagglutination caused by T-activation was detected and a bone marrow biopsy revealed that marked erythropoiesis with normal myelopoiesis was taking place.

Negative or normal findings were obtained for the following: Schumm's test, hemoglobin electrophoresis, fetal hemoglobin, G-6-PD, red blood cell sickling, Donath-Landsteiner test, L.E. test, rheumatoid factor, and blood urea. A blood culture was not done, but no pathogenic organisms were isolated from the patient's sputum, her urine was clear, and a second chest X-ray showed that her pneumonia was clearing. Anti-ampicillin antibodies were not detected in any of her serum samples.

In view of the apparently positive direct antiglobulin test, autoagglutinins, anemia, and jaundice, she was considered to have autoimmune hemolytic anemia. Prednisone therapy (45 mg daily) was commenced. The patient's condition soon began to improve and, after several days, the average size of her T-activated red blood cell agglutinates was seen to have decreased. Ten days after her crisis, her reticulocyte count was 45 per cent. Fifteen days later, she was discharged fit and well with a hemoglobin of 9.8/100 ml and 16 per cent reticulocytes. She has not returned.

Serological Studies

The patient's red blood cells were seen to be agglutinated in mixed field patterns when the direct antiglobulin test done during her crisis period was examined microscopically. The agglutinates were present in saline suspensions of her red blood cells prepared from clotted samples of her blood and were not caused by anti-T in the antiglobulin reagent. A second blood sample was taken with a warmed syringe and needle into anticoagulant and maintained at 37 C. Although there was some evidence of rouleaux on direct examination under the microscope, no agglutinated red blood cells were seen at this stage. However, when the drop of blood on the microscope slide was mixed with a drop of saline by gentle rotation, agglutinates appeared and persisted.

Despite the presence of the agglutinates, the patient's red blood cells were successfully tested with selected avid blood grouping reagents of adult origin. Anti-P_1 and anti-Lu^a were excluded as they are known to agglutinate red blood cells in mixed field patterns. The results were confirmed where possible with cord serum containing blood group antibodies and with aged reagents both of which are known to lack anti-T ag-

glutinins.[11,18,27] Adult A_1B serum was included throughout as a negative control. The patient's groups were as follows: O ss ccDee hr^s + K− Kp(a−) Fy(a−) I+.

The H antigen of the cells was not increased in strength when compared with normal group O cells in parallel titrations, and no agglutination other than mixed field agglutination was observed when the cells were tested in saline with incomplete anti-c, anti-e and D^u serum. Pretreatment of the cells with a 0.25 per cent solution of ficin in buffered saline for 10 minutes at 22 C, followed by washing them three times with saline, or simple addition of 0.5 per cent bromelin solution to the test mixture, removed all evidence of mixed field agglutination. The ABO and Rh groups of the cells were then readily confirmed using reagents suitable for grouping enzyme-treated cells. The cells were also found to be Le (a−b−).

No agglutination other than mixed field agglutination was detected when saline suspensions of the patient's cells were tested with seven human anti-M and two human anti-N reagents. However, strong agglutination (not in mixed fields) occurred with three human anti-M, one human anti-N, and a commercial (rabbit) anti-M. These sera were shown not to contain anti-I in tests with T-activated I+cells.[19] Two different batches of an anti-N lectin also produced strong agglutination. Her cells were also strongly agglutinated (titer 512 at 22 C) by a saline extract of raw peanuts prepared according to the method of Bird[3] and not by an extract of the seeds of Dolichos biflorus.[6] These results indicate that they were T-activated and not Tn-activated. Control T-activated red blood cells made by treating normal cells with receptor destroying enzyme (R.D.E.; Wellcome Reagents, England) were agglutinated (titer 512 at 22 C) by the peanut extract, while untreated cells were not agglutinated. The reactions of the patient's cells were not enhanced by treatment with papain,[7] and enhanced agglutination was not observed with anti-Sd^a, showing that they were not Tk-activated or Cad-positive.[5] However, when suspended in a commercial 1 per cent solution of protamine sulphate, known to be a suitable substitute for Polybrene,[16] which was unobtainable, they were seen to be aggregated weakly. Control normal cells were aggregated strongly (+++) and R.D.E.-treated cells were not aggregated by the solution of protamine sulphate.

An eluate was made by washing some of the cells from the patient's anticoagulated blood sample (which had been maintained at 37 C) six times in large volumes of saline and then agitating them in a small amount of saline for five minutes at 56 C. The eluate agglutinated (titer 8 at 10 C) R.D.E.-treated cells in saline at 22 C and did not agglutinate a panel of eight cell samples containing all the more common blood group antigens, in saline at 22 C, by enzyme, or by indirect antiglobulin techniques. The plasma from the same sample, and serum separated from samples of her blood allowed to clot normally at room temperature (22 C), also agglutinated the R.D.E.-treated cells and did not agglutinate the panel of eight cell samples in saline at 22 C, by enzyme or by indirect antiglobulin techniques. These tests showed that her plasma, serum, and red blood cell eluate all contained only anti-T. It was subsequently established that the titer of the anti-T in her plasma separated at 37 C was 256 when tested at 4 C, 128 when tested at 10 C, 64 when tested at 22 C and nil when tested at 37 C.

In a further blood sample, received from the patient during the recovery period following her hemolytic crisis, the spontaneously appearing agglutinates were seen to be very much smaller in size. Unfortunately, it was not possible to obtain a sample after she had left the hospital to confirm that her phenotype was MN and show that the T-activated cells had eventually completely disappeared.

Discussion

In previous cases of red blood cell polyagglutination caused by T or Tn-activation in adults, no evidence of anti-T or anti-Tn has

been demonstrated in the patient's plasma at the time. It has been assumed that the antibody was absorbed onto the patient's cells or was in some way inhibited by immune tolerance or immune paralysis. Activation was seldom associated with red blood cell hemolysis *in vivo*. Hemolysis in children was shown to be due either to anti-T in transfused plasma or to a bacterial infection. Our patient's T-activated red blood cells were clearly agglutinated by the anti-T in her own plasma. This provides new evidence to support the view held by Rickard, *et al.*[26] that T-activated red blood cells, the patient's own anti-T, and severe red blood cell hemolysis and anemia *in vivo* may be causally related.

The patient's cells, which appeared in titrations with peanut anti-T to be well T-activated, showed indications that activation was not complete. The cells were agglutinated by some, but not all of the human anti-M and anti-N reagents used. This was not due to variation in the strength of these antibodies. A solution of protamine sulphate caused weak aggregation and H antigen was not increased in the activated cells. They were not agglutinated in saline by incomplete Rhesus antibodies. Partial T-activation may have been due to the recent onset of the patient's hemolytic anemia, or to the administration of ampicillin which may have already succeeded in suppressing a possible causative organism. However, Bird[7] has reported that the removal of only very small amounts (5 to 7%) of sialic acid from the red blood cell membrane was sufficient for the cells to react strongly with peanut anti-T.

Since no bacteria were isolated from the fluid in the pelural cavity in our patient and her blood was not cultured, the identity of the neuraminidase-producing organism which caused her red blood cells to become T-activated is not known. Her elevated temperature, leukocytosis, and lung signs indicated that she had a pulmonary infection. In retrospect, it seems likely that the autoagglutination observed in her early blood films was due to T-activation. During the first seven days after hospitalization, her condition appeared to improve under ampicillin treatment and no clinical signs of increased red blood cell destruction were seen. However, the extent of the anemia discovered during the crisis period, and the hematological and biochemical findings at this stage indicate that extravascular hemolysis had taken place. In the absence of evidence of other auto-antibodies or of anti-ampicillin antibodies which might have produced a similar effect, we conclude that, though the T-activation of the cells appeared to be incomplete, the anemia, T-activated cells, and anti-T were causally related. A rapid improvement took place after prednisone therapy was commenced, and the corresponding reduction in the size of the agglutinates showed that T-activation also decreased as the patient recovered.

No studies were made to determine whether the patient's T-activated cells could be agglutinated by specific anti-IgM, anti-IgG, or anti-complement reagents since they had not been agglutinated by a broad-spectrum antiglobulin reagent in the initial tests. Similar difficulties associated with the detection of red blood cell bound IgM antibodies with antiglobulin reagents have often been experienced by other workers. Instead, the low thermal optimum was taken to indicate that her anti-T was an IgM antibody.

The mechanism by which the red blood cell destruction took place *in vivo* in our patient is difficult to explain. There was no evidence of intravascular hemolysis which might have indicated the presence of an IgG anti-T or complement coating her cells. Anti-T does not normally fix complement. This view is consistent with the recovery of anti-T in the eluate made from our patient's cells maintained at 37 C. The possibility exists that her T-activated cells may have been eliminated because they were damaged by physical rather than immunologic means by the removal of sialic acid, and agglutination by her IgM anti-T may then have occurred merely as an incidental phenomenon. Alternatively, the membranes of her cells

may have been altered in such a way that her anti-T was only loosely attached to them, allowing easy recovery in the eluates. This is the more likely, since it explains the normal absence of anti-T in adults with T-activated cells. We believe that the anti-T in our patient may have been unusually well developed.

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P. Moores, B. Sc. (Bristol), M.T. (S.A.), Scientific Officer, Natal Blood Transfusion Service, 149 Prince Street, Durban, South Africa.

D. Pudifin, M.B., Ch.B. (U.C.T.), F.C.P. (S.A.), M.R.C.P. (U.K.), Senior Lecturer, Department of Medicine, University of Natal, Durban, South Africa.

P. L. Patel, M.B., Ch.B. (Natal), F.C.P. (S.A.), M.R.C.P. (London), Lecturer, Department of Medicine, University of Natal, Durban, South Africa.

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Haemoglobin M-Hyde Park associated with polyagglutinable red blood cells in a South African family

A. R. Bird, P. Kent,* P. P. Moores* and T. Elliott *Department of Haematology, Groote Schuur Hospital and University of Cape Town, South Africa and *Natal Blood Transfusion Service, Durban, South Africa*

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Summary. Twelve of 35 members tested in a large ethnically-mixed South African family were found to have both haemoglobin M type Hyde Park and persistent polyagglutinable red blood cells. The characteristics of the polyagglutination have not been recorded previously. The cells of affected family members were not agglutinated by *Arachis hypogea*, *Dolichos biflorus* or *Salvia sclarea*, but were agglutinated weakly by *Salvia horminum* and BSII (GSII) and reacted strongly with *Glycine soja* and *Sophora japonica* lectins. BSI (GSI) lectin agglutinated the group A but not the group O cells. The N and MN cells were agglutinated more strongly than normal by *Vicia graminea*, other anti-N lectins and human anti-N but the M and MN cells reacted as expected with human anti-M. The name 'Hyde Park' is provisionally suggested for this type of polyagglutination, although it appears unlikely that the evidently complete association between the polyagglutination and the variant haemoglobin is the result of a single genetic mutation. More likely, the connection has a post-genetic origin, perhaps showing that bonds, possibly affected adversely by precocious senescence, normally occur between the haemoglobin and α-sialoglycoprotein molecules in red blood cells.

Methaemoglobinaemia caused by inheritance of variant haemoglobin (Hb) genes is relatively rare. The disorder was first clearly described by Jaffé & Heller (1971) in four generations of a family. The biochemical structure of the abnormal haemoglobin was later determined and the haemoglobin named Hb M. Five variants are now known, the major consequence of all of which is congenital cyanosis. In four, amino acid substitution has occurred in the haem pocket. This affects the haem–globin bond adversely but molecular stability is maintained by new bonds being formed between the substituted amino acid and the haem iron. The abnormal haemoglobin remains in the ferric state, however, as it is incapable of being reversibly oxygenated. In Hb M-Iwate and M-Hyde Park, the proximal, and in M-Boston and M-Saskatoon the distal histidine amino acids have mutated and the haem iron is bonded instead to the phenolic side chain of the tyrosine amino acid replacement. In M-Hyde Park and M-Saskatoon a β-globin chain, and in M-Iwate and M-Boston an α-globin chain is affected. In M-Milwaukee, the amino acid valine at position 67 on a β-globin chain has mutated to glutamic acid. Nevertheless, the carboxyl group of this residue is close enough to the haem iron for a stable bond to be formed (Winslow & Anderson, 1983).

Polyagglutinability reflects alterations that occur in the membranes of red blood cells as the result of aberrant erythropoiesis or bacterial or viral activity, and the term 'polyagglutination' refers to the fact that the cells are agglutinated by many normal ABO compatible sera and give positive or enhanced positive results with certain seed and other lectins. Ten different forms are known. They are T, Tn, Tk, Th, Tx, Cad, acquired B, VA, HEMPAS and NOR.

We present the clinical, haematological and serological findings in a large kindred of mixed ethnic origin in which 12 members are mildly cyanosed and have haemoglobin M, type Hyde Park. This is the third variant methaemoglobin to be described from South Africa (Bernstein, 1965; Botha *et al*, 1967) and the third report to be published of M-Hyde Park occurring in a large family (Becroft *et al*, 1968; Efremov *et al*, 1974; Heller *et al*, 1966; Shibata *et al*, 1968; Stamatoyannopoulos *et al*, 1976). It is the first report of polyagglutinable red blood cells appearing in close association with a variant haemoglobin.

CASE REPORT

The propositus, who is of mixed Black/White/Khoisan descent, was first noticed when a donation of his blood was seen to be almost black in colour. A variant haemoglobin was found on haemoglobin electrophoresis and a family study begun. At the same time, weak positive results obtained in compatibility tests with the red cells of the propositus and the sera of several possible recipients suggested that his ABO phenotype was not O but A_{bantu}. Reference laboratory tests showed instead that his cells were polyagglutinable.

Thirteen family members (one has since died) are known to

Correspondence: Dr A. R. Bird, Haematology Laboratory, Groote Schuur Hospital, Observatory, 7925 Cape Town, South Africa.

have or to have had the variant haemoglobin. The propositus (II-22, Fig 1) is 40 years of age, active and hard-working. He complains only that during the past 5 years skin sores have taken longer to heal and he recovers more slowly from influenza. By 1979 he had given 12 blood donations but has never received a blood transfusion himself. Recent examination showed that his lips and fingernails are mildly cyanosed and that his spleen is enlarged to 4 cm below the costal margin. He has a normal male karyotype. His mother (I-8) died aged 59 years from disseminated intravascular coagulation. Four other similarly affected male family members (II-17, -18, -24, III-16), aged from 10 to 38 years are all healthy, have no physical complaints and have not received blood transfusions. With the exception of II-24, who has slightly cyanosed lips, medical examination showed nothing abnormal. By contrast, six similarly affected female family members (II-14, -15, -25, -27, III-14, -17), aged from 11 to 36 years, complained of occasional tiredness, sometimes associated with dizziness and headache. These symptoms in the older women had been exacerbated by pregnancy. All six females had received one or two blood transfusions in the past for anaemia. Examination showed mild cyanosis of the lips but no splenic enlargement. Matched as closely as possible for age and height, the affected males weighed less than the unaffected males. The affected females, however, weighed more than the unaffected females.

MATERIALS AND METHODS

Full blood counts were done with a Coulter S Plus II counter, and other routine haematological studies according to standard techniques. The haemoglobin was examined by starch gel electrophoresis using Tris–EDTA–borate buffer pH 8·6 and with phosphate buffer pH 7·1 after conversion to the met-form with potassium ferricyanide (White & Frost, 1984). The haemoglobin spectral analysis was conducted at wavelength 450–700 nm in a Pye-Unicam double beam spectrophotometer. The total methaemoglobin production was measured by the method of Evelyn & Malloy (1938) and the Hb A_2 and Hb F levels assayed by the methods of Huisman *et al* (1975) and Pembrey *et al* (1972) respectively. The NADH-methaemoglobin reductase levels were measured according to the method of Beutler (1984). The haemoglobin stability was assessed by the isopropanol and heat tests (Carrell & Kay, 1972; Dacie *et al*, 1969). The variant haemoglobin was isolated by DEAE-cellulose and the abnormal β-globin chains by carboxymethylcellulose chromatography. The tryptic peptides were analysed by reversed phase, high-pressure liquid chromatography (HPLC) (Wilson *et al*, 1979).

The family blood samples were examined serologically within 24 h of receipt. Standard manual techniques were used. The human reagents were either from commercial sources, standardized local donations or gifts from colleagues in other centres. Known positive and negative control red blood cells were included with every batch of tests. The bromelin (0·5% bromelin powder in phosphate-buffered saline, pH 5·5), ficin (0·25% in phosphate-buffered saline, pH 7·3) and protamine sulphate solutions were from commercial and local sources.

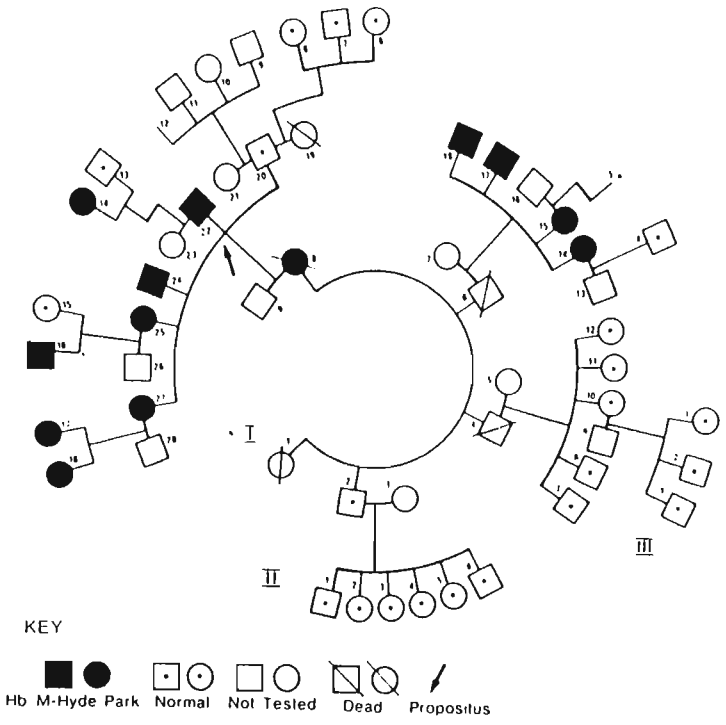

Fig 1. Family pedigree.

RESULTS

Haematology

The haemoglobin levels of all but one of the family members with Hb M-Hyde Park tested fell within the normal range for their ages (Table I). The exception is I-8 who had a markedly macrocytic anaemia. At the time of testing, she was suffering from disseminated intravascular coagulation and this subsequently caused her death. The blood samples of the other affected family members showed mild macrocytosis and reticulocytosis. No Heinz bodies were detected but the isopropanol and heat tests caused precipitation in the haemolysates of two members. The NADH-methaemoglobin reductase levels in the red blood cells of these members were normal.

Electrophoresis and spectral analysis

A distinct band migrating just ahead of the Hb A_2 band was identified on haemoglobin electrophoresis in all the affected family members tested. Electrophoresis of the oxidized haemolysate also showed a band at pH 7·1, just cathodal to that for methaemoglobin A. Prior to staining, this band had a blue-green colour. The spectrophotometric scan of the oxidized haemolysate produced an obvious plateau between 580 and 630 nm whereas methaemoglobin A normally provides a trough in this region (see Fig 2).

Chromatography and structural analysis

When separated by reversed-phase HPLC, the chromatogram of the tryptic peptides showed that β T-10 occurred at an abnormal position: its amino acid composition was (normal values in parentheses): Asp 0·94 (1), Thr 1·62 (2), Ser 1·04 (1), Glu 1·04 (1), Gly 1·08 (1), Ala 1·17 (1), Leu 1·88 (2), Tyr 0·92 (0), Phe 0·88 (1), His 0·10 (1), Lys 1·01 (1), cysteine present.

The His-Tyr substitution seen in this peptide is typical of Hb M-Hyde Park or $\alpha_2\beta_2$92(F8) His→Tyr.

Serology

The red cells of the family members with Hb M-Hyde Park tested were moderately or were not at all aggregated by protamine sulphate solution. Their cells were agglutinated weakly, often in mixed field patterns, by many samples of ABO compatible human sera irrespective of whether these sera contained anti-A. These reactions were enhanced by bromelin and ficin in one-stage enzyme tests. Neither cord sera nor monoclonal anti-A agglutinated the O cells, and human anti-A was not recovered in eluates following exposure of the cells to a potent example of this antibody. The serum of the propositus contained weak anti-T, and his anti-A, B reacted more strongly in titrations with A_1 than with B cells. His saliva contained H but no A or B substances. Commercial (rabbit) anti-M and anti-N reacted normally with the family members' cells. However, in titrations with human anti-N and the lectins *Vicia graminea, Molucella laevis* and *Bauhinia purpurea alba*, the type N and MN affected members' cells were agglutinated more avidly and to higher titres than the control N cells (Table II). In contrast, the type M and MN affected members' cells were agglutinated to the same titre as the control M cells by human anti-M. The family

Table I. Haematological investigations on blood samples from members of the family with Hb-M Hyde Park

Fig 1 No.	Age*/ sex	Hb (g/dl)	PCV	RBC (10^{12}/l)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Methb. (%)	Hb A_2 (%)	Hb F (%)	Retics (%)	Hb electro.† (band in A_2 region %)	Isopr. ppt‡	Heat ppt§
I-8	59/F	7·3	0·25	1·72	145	42	29			3·2	15·7	10		
II-2	Ad/F	13·7	0·44	4·59	96	30	31			1				
II-3	Ad/F	16·7	0·46	6·10	75	27	36			1		Normal		
II-14	24/F	11·8	0·33				36			5·2	2·5	10·4		
II-15	9/F	13·2	0·44	4·20	105	31	30			2·6		8·2		
II-17	19/M	14·3	0·43	4·77	90	30	33			2·3	6·2	18		
II-18	15/M	13·5	0·44	4·25	103	32	31			4·2	4·0	13		
II-20	38/M	14·8	0·43	4·35	99	34	34			1		Normal		
II-22	40/M	13·4	0·42	3·81	110	35	32	1·9	6·1	2·4		18	Pos	Pos
II-25	28/F	12·1	0·41	3·92	104	31	30			2·5	2·8	14·5		
II-27	30/F	13·1	0·43	4·26	101	31	32	5·0	6·0	4·5		12	Pos	Pos
III-4	2/M	12·1	0·39				31			1	1·2	Normal		
III-6	17/F	12·8	0·42	3·96	106	32	30			1		Normal		
III-7	15/M	13·3	0·41	4·22	97	32	32			1		Normal		
III-8	13/F	11·9	0·38	3·94	96	30	31			1		Normal		
III-9	8/M	13·4	0·43	4·35	99	31	31			1		Normal		
III-14	4/F	12·8	0·39	4·40	90	30	33			3·9	4·6	10		
III-15	17/F	13·1	0·43	4·95	87	27	30	1·2	2·2	1	0·4	Normal	Neg	Neg
III-16	2·5/M	10·7	0·35	3·61	97	30	31			8·4	2·5	11		
III-17	3/F	11·4	0·34	3·46	98	32	34			2·8	3·4	12		

* Age in years when examined. † Haemoglobin electrophoresis at pH 8·6. ‡ Isopropanol precipitation § Heat precipitation.

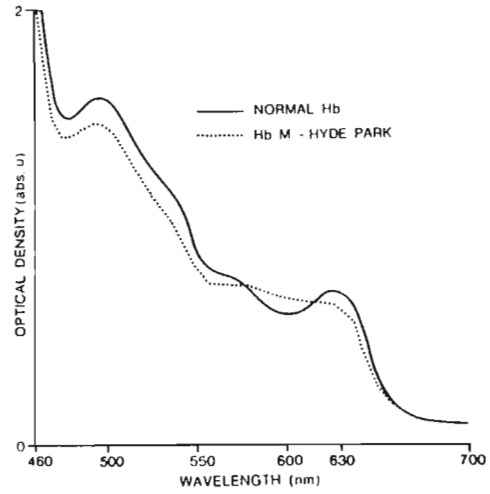

Fig 2. Spectrophotometic tracing of oxidized haemolysates.

members' cells were not agglutinated by anti-M_1, -M^g, -Henshaw, -Mur, -Hut or -Dantu, and their S, s and U antigens were expressed normally. The reactions of the cells with reagents in other blood group systems, interpreted with difficulty owing to the polyagglutination, are available on request.

Table III shows the results obtained on characterizing the polyaggluntinability of the affected family members' cells with various lectins. *Glycine soja* agglutinated the cells strongly and *Salvia horminum* weakly. Mixed field agglutination, indicative of Tn cells, was not detected in these tests. The cells differed from all those with other types of polyagglutination in that *Arachis hypogea*, *Dolichos biflorus* (with group O cells), *Salvia sclarea* and *Phaseolus limensis* lectins failed to agglutinate them. The group O cells were also not agglutinated by BSI (GSI) but BSII (GSII) lectin, which gave 4 + strong agglutination with group A cells, agglutinated the group O cells only microscopically. In titrations with non-immune rabbit serum, the affected members' cells reacted to higher titres than the controls. The I, H and Sd^a antigens of their cells were not enhanced but 4 + strong reactions (titre equal to cord cells) were obtained with anti-i reagents. None of the affected family members' cells reacted in saline tests with incomplete anti-Rh sera. Although fresh blood samples have been tested repeatedly during the past 7 years, no alterations were observed in these findings.

DISCUSSION

Haematology

The findings in this family exhibiting interesting diagnostic, biochemical, serological and genetic features. They underline the problems inherent in identifying disorders due to haemoglobin M and may provide new information about the mechanism of red cell damage brought on by oxidant stress. Clinically, haemoglobin M is relatively easy to detect as the patients are cyanosed. The cyanosis, however, is not a dark blue but a lavender blue colour (Lehmann & Huntsman, 1974). In persons with dark brown skins it is therefore difficult to detect and the cyanosis is sometimes seen only during stress as, for example, in a child crying. Nevertheless in one patient the cyanosis was sufficiently obvious to suggest congenital heart disease (Stamtoyannopoulos *et al*, 1976). The total methaemoglobin content of the red blood cells may be misinterpreted as the abnormal spectral and cyanide reactivities invalidate the results. Laboratory diagnosis therefore depends upon demonstrating abnormal met-converted haemolysate spectral patterns. Specific absorption peaks are described for each Hb M variant but, as seen in this family, the pattern with oxidized haemolysates in Hb M-Hyde Park is a plateau or slight elevation of the scanning trace in the 580–630 nm region. Standard haemoglobin electrophoresis at alkaline pH is usually not suitable for demonstrating haemoglobin M, but another characteristic of Hb M-Hyde Park is the distinct band appearing near that for Hb A_2 by this technique. A further characteristic is the blue-green band seen just cathodal to methaemoglobin A on electrophoresis of the metform at pH 7·1. Both haemoglobin M-Hyde Park and M-Saskatoon are also associated with haemolysis. This

Table II. Titrations of human anti-N and anti-N lectins showing enhanced reactions of Hyde Park cells compared with normal control red cells

Reagent	Cells	Blood group	Reciprocals of titres 1	2	4	8	16	32	64	128	256	512	1024
Human anti-N	Hyde Park	O, N	4	4	3	3	3	2	1	(3)	–	–	–
	Control	O, N	3	3	2	1	–	–	–	–	–	–	–
Bauhinia purpurea	Hyde Park	O, N	4	4	4	4	4	3	2	1	(3)	(1)	–
	Control	O, N	1	1	1	(3)	(2)	–	–	–	–	–	–
Vicia graminea	Hyde Park	O, N	4	4	3	2	1	–	–	–	–	–	–
	Control	O, N	4	4	2	1	–	–	–	–	–	–	–
Moluccella laevis	Hyde Park	O, N	4	4	4	4	3	3	2	1	(3)	(1)	–
	Control	O, N	4	3	2	1	(3)	(2)	—	–	–	–	–

Technique: saline at ± 22°C; dilutions made in saline; tests centrifuged gently before being read.
Key: Macroscopic: 4, 3, 2, 1, –; microscopic: (3), (2), (1), —.

Table III. Reactions of lectins and other reagents with Hyde Park red cells

	Hyde Park		Controls		Reactions of other polyagglutinable cells								
Red cells	O, N	A_1, N	Pos.	Neg.	T	Tn	Tk	Th	Tx	Cad	Acq B	NOR	HEMPAS
Arachis hypogea	–	–	4	–	+	–	+	+	+	–	–	–	–
Dolichos biflorus	–	4	4	–	–	+	–	–		+		–	–
Glycine soja	4	4	4	–	+	+	–	–	–	+	–	–	+
Sophora japonica	2	4	1	–	+	+				+			
Salvia horminum	1	1	4	–	–	+	–	–	–	+	–	–	–
Salvia sclarea	–	–	4	–	–	+	–	–	–	–	–	–	–
Vicia graminea	4	4	4	–	+								
Anti-M (human)	(2)	(2)	4	–	–								
Anti-N (human)	4	4	4	–	–								
BS I	–	4	2	–		+							
BS II (GS II)	(1)	1		–	–	–	+	–	–	–	–	–	–
Anti-Sd^a	(1)	(1)	1	–						+			

Twelve affected family members were tested. They comprised one group A_2, two group A_1 and nine group O. Three members were genetically type M, four MN and five N.

Grading of results: Macroscopic: 4, 3, 2, 1, –; microscopic: (3), (2), (1), (±), –.

manifestation of membrane instability was confirmed in Hb M-Hyde Park by X-ray crystallographic analysis which showed loss of β-globin groups from 20–30% of the molecules in crystalline preparations (Greer, 1971). The instability is, however, moderate and the haemolysis mild and compensated for easily.

Serology

Polyagglutinable red blood cells do not appear to have been observed previously in association with a variant haemoglobin. In this family, all 12 tested members with haemoglobin M-Hyde Park have the membrane defect which is absent in all of the 23 tested members with normal haemoglobin. The haemoglobin–polyagglutination association is therefore complete, and the polyagglutinability is unlikely to have been caused by *in vivo* bacterial or viral activity. The pattern of reactions obtained with the polyagglutinable cells (Table III) differs from all others so far recorded (Issitt, 1985, pp. 456–473). Since neither the *Arachis hypogea* (anti-T) nor *Dolichos biflorus* (anti-A_1 + Cad) lectins agglutinated the cells of the affected group O family members, neither β-galactose (exposed on T-sensitized cells) nor N-acetyl-D-glactosamine (the group A determinant) appears to have been available on the cells for antigen binding. The possibility that the polyagglutination was of T, Th and Tx types is therefore unlikely, and of type Tk uncertain, owing to the weaker than expected positive results obtained with BS II (GS II) lectin. In addition, Tn exposure as the cause of the polyagglutination is questionable since (i) *Salvia sclarea* failed to agglutinate either the A or O affected members' cells; (ii) BS I (GS I) did not agglutinate the O cells; (iii) *Salvia horminum* reacted with the A and O cells too weakly; (iv) neither *Glycine soja* nor *Sophora japonica* reacted with the group A or O cells in mixed field patterns, and (v) the group O cells were weakly agglutinated by human sera of all ABO groups and not only by those containing anti-A. Nevertheless, *Salvia horminum* lectin is known to be capable of complexing with N-acetyl-D-galactosamine provided that the molecular configuration is suitable, and furthermore BSI (GS I) lectin reacted strongly with the cells of the affected group A family members. Polyagglutination due to Cad and HEMPAS was excluded as the cells of the affected members had normal strength Sd^a, enhanced N antigens and aggregated normally with protamine sulphate. The association with acquired B and NOR was excluded since these cells are characteristically non-reactive with *Glycine soja*.

Despite this uncertain interpretation, loss of N-acetylneuraminic acid (NeuAc) from the red cell membranes of the affected family members has clearly occurred. This was shown by the strong reactions of their cells with *Glycine soja* and *Sophora japonica* lectins, and weak or no aggregation in protamine sulphate solution. The enhanced N antigen contrasts markedly with the weak M and N antigens normally detected in other types of polyagglutination (Issitt, 1985, pp. 456–473). The M antigen did not appear similarly enhanced but the prozone-like appearance provided by the MM cells in titrations with *Bauhinia purpurea alba* suggests that the cells were nonetheless polyagglutinable. Possibly the human anti-M reagents that were used are unable to recognize membrane NeuAc deficiency. Judd *et al* (1979) reported that whereas some MN system antibodies react with red cells only when the full complement of NeuAc is present, other sera react more strongly when NeuAc is absent or has been removed, while further reagents show no preference. The antigens M and N are identified biochemically solely by different amino-acids situated at positions 1 and 5 at the NH_2-terminus of alpha-sialoglycoprotein molecules (Issitt, 1985, pp. 338–341; Low *et al*, 1985) and polyagglutination occurs when the alkali-labile oligosaccharide units attached at positions 2, 3 and 4 (and possibly more) of these chains are in some way altered. These alterations, which affect the steric configuration of the molecule, are usually caused by bacterial or viral activity but may also be the result of an inherited mutation. As the polyagglutination in this family is of a so far unrecognized type, and occurred only in the family members

with the variant haemoglobin, we propose that it be named type 'Hyde Park'. This would be confirmed to be an appropriate nomenclature if other unrelated persons with the same haemoglobin were also discovered to have similarly polyagglutinable cells.

The close association between the variant haemoglobin and polyagglutinability in this family may provide further insight into mechanisms of oxidant-induced haemolysis. M-Hyde Park is a mildly unstable haemoglobin and its denaturation in *vivo* results in hemichrome (Heinz body) formation, although this may not always be observable. Low *et al* (1985) reported that hemichromes show high affinity for the cytoplasmic domain of band 3 protein, cross-linking it into clusters. The clusters provide recognition sites for antibodies directed at senescent red cells and hasten their removal from the blood. Galili *et al* (1985) discovered large amounts of naturally occurring IgG anti-α galactosyl antibodies in normal human sera and showed that they bind to senescent and thalassaemic cells, supporting this view. Possibly, in our family, hemichromes appear earlier than usual and affect band 3 protein structure adversely. Since there is support for believing that band 3 and α-sialo-glycoprotein form complexes in intact red cell membranes (Anderson & Lovrien, 1984; Dahr, 1986), 'Hyde Park' polyagglutination may be a product of normal red cell denaturation occurring in unusual circumstances.

Whether or not the red cells in disorders caused by other forms of oxidant-induced haemolysis will exhibit the same or a similar form of polyagglutination remains to be seen.

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Paper 46

Enhanced reaction with *Vicia graminea* lectin and exposed terminal N-acetyl-D-glucosaminyl residues on a sample of human red cells with Hb M-Hyde Park

M. J. KING, Y. W. LIEW, P. P. MOORES, AND G. W. G. BIRD

A sample of polyagglutinable red cells was obtained from a healthy individual (group O, N) possessing a hemoglobin (Hb) variant called Hb M-Hyde Park. The sialic acid content of the individual's red cells is 90 percent of normal, and his cells are agglutinated by monoclonal but not lectin anti-Tn, a panel of lectins specific for N-acetylgalactosamine (or galactose), and N-acetylglucosamine. Enhanced agglutination reactions were obtained with *Vicia graminea, Ulex europaeus,* and human anti-I and -i. Using various enzyme treatments and different methods of labeling cell surface components, two defective cell membrane sites have been identified: one associated with the O-linked oligosaccharides on sialoglycoproteins and the other associated with exposed N-acetylglucosaminyl residues located on membrane components of apparent molecular weights 88,000 to 130,000 and 46,000 to 73,000 (probably the Band 3 and Band 4.5 regions, respectively).
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RED CELL (RBC) POLYAGGLUTINATION represents an alteration in the RBC surface property that can be either transient and attributable to bacterial or viral infection (e.g., T, Tk, or acquired B),[1] persistent (e.g., Tn), or permanent and attributable to somatic or hereditary changes (e.g., HEMPAS or NOR).[2,3] The affected RBCs are polyagglutinable with normal adult, ABO-compatible sera, and various lectins have been used to classify the different types of polyagglutination resulting from dissimilar etiology.[3] Recently, Bird et al.[4] reported a type of polyagglutination found over a period of 7 years with the RBCs of an apparently healthy blood donor of mixed race and of 12 affected members of his family, all of whom have a methemoglobin variant, type hemoglobin (Hb) M-Hyde Park. Moreover, the blood samples of the affected individuals showed mild macrocytosis and reticulocytosis. Their cells were agglutinated strongly by *Glycine soja* and weakly by *Salvia horminum* lectins. A weaker reaction than that of Tk-activated red cells was obtained with the lectin from *Griffonia simplicifolia* II (GS II), formerly *Bandeiraea simplicifolia* II (BS II). RBCs from the affected group O family members were not agglutinated by *Arachis hypogaea* or *Dolichos biflorus.* The affected group A or O members' RBCs were not agglutinated by *Salvia sclarea,* indicating a lack of exposed Tn antigen. In titrations using human anti-N, *Vicia graminea, Molucella laevis, Bauhinia variegata candida,* and *Bauhinia purpurea alba,* marked enhancement in the agglutination of the affected group N and MN family members' cells was obtained.

The present study has extended the preliminary investigation of Bird et al.[4] by carrying out further serologic tests using proteases and endo-β-galactosidase to distinguish between the reactions with specific lectins. Biochemical methods were also used to probe the possible alterations in the RBC surface carbohydrate structures and membrane protein components.

Materials and Methods

Fresh blood samples from the propositus (group O,N) and a normal control (group O, N, to be called natal control) were received by air shipment from South Africa within 4 days of the blood's having been drawn into CPD anticoagulant. All the tests were completed within 1 week of the arrival of the blood in England. After having been stored at −20°C in glycerol, the propositus's RBCs that had been recovered by dialysis compared rather poorly with the natal control red cells. This suggested that the membranes of the propositus's RBCs were slightly more fragile than normal. A sample of Tn RBCs (J.D.) frozen in glycerol was obtained through an international exchange scheme (SCARF). Hexadimethine bromide (Polybrene) was obtained commercially (Aldrich Chemical Company, Dorset, UK), as were the lectins GS II and *Vicia villosa* B_4 (Sigma Chemical Company, Poole, UK). The lectins *Bauhinia purpurea, Limulus polyphemus,* and *Helix pomatia* were gifts; 1 g of seeds was extracted with 10 ml of phosphate-buffered saline (PBS) from each of the following lectins, which were used without further purification: *A. hypogaea, D. biflorus, Salvia sclarea, S. horminum, Vicia cretica, V. graminea, Vicia hyrcanica, Leonurus cardiaca, Medicago disciformis,* and *Ulex europaeus.*

Endo-β-galactosidase concentrate (free from sialidase and proteases) was prepared by chromatography of a culture supernanant of *Bacteroides fragilis* (provided by Dr. D.J. Rogers, Portsmouth Polytechnic, Portsmouth, UK) on a column of dextran (CM-50 Sephadex, Milton Keynes, UK). The antisera and monoclonal anti-A, -B, and -N were the routine blood grouping reagents. The murine monoclonal

From the Blood Group Reference Laboratory, Oxford, England, and the Natal Blood Transfusion Service, Durban, South Africa.
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antibodies (MoAbs) (obtained from Dr. D.J. Anstee, Bristol, UK) were: sialic acid-dependent and -independent anti-N (BRIC 33, BRIC 115, BRIC 120, BRIC 123, and BRIC 157), anti-Tn (BRIC 111, BRIC 113, and BRIC 114), and R1.3. The R1.3 antibody recognizes an epitope on the identical N-terminal region of the alpha and delta sialoglycoproteins, and its reaction with RBCs is sensitive to neuraminidase treatment.[6] ^{14}C-methylated protein molecular weight standards (MW 14.3K to 200K) and radiochemicals were obtained commercially (Amersham, Aylesbury, UK).

Enzyme treatments of red cells

For endo-β-galactosidase treatment (i.e., in vitro Tk activation) 100 μl of enzyme concentrate was incubated with 50 μl of packed RBCs at 37°C for 3 hours. For protease treatment 100 μl of packed RBCs was incubated at 37°C with 400 μl of trypsin (2.5 mg/ml; Type XIII, Sigma) for 30 minutes or with 100 μl of papain (10 mg/ml; BDH Limited, Poole, UK) for 15 minutes. For neuraminidase treatment 100 μl of packed RBCs was incubated with 200 μl of neuraminidase (0.02 U/ml; Type VI, Sigma) in PBS at 37°C for 1 hour. The supernatant from the neuraminidase-treated cells was retained for the quantitation of sialic acid.[7]

Labeling of cell surface components

On intact RBCs, terminal N-acetylglucosaminyl residues were labeled with ^{14}C-galactose by incubation of 30 μl of packed RBCs with uridine diphosphate (UDP)-[U-^{14}C]-galatose (2×10^5 cpm, specific activity 297 mCi/mmol) with 300 mU of bovine milk β-galactosyltransferase (IU/ml, Sigma) in 110 μl of Buffer A (50 m*M* Tris-HCl buffer [pH 7.8], 15 m*M* $MnCl_2$, and 150 m*M* NaCl) at 37°C for 6 hours.[8] In incubation with unlabeled UDP-galactose (0.5 m*M*), 100 μl of packed RBCs and 90 mU of β-galactosyltransferase in 350 μl of Buffer A were used. The treated cells were titrated with lectins from GS II, *V. graminea*, *V. hyrcanica*, *U. europaeus*, and human anti-I and -i. Standard serologic techniques were used, and the scoring system was that of Marsh.[9] Sialoglycoproteins were tritium-labeled by periodate oxidation and sodium boro [^{3}H]hydride reduction,[10] and membrane proteins were iodinated by the method using lactoperoxidase.[11] After sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 percent gels,[12] ^{14}C- and ^{3}H-labeled membrane components were visualized by fluorography[13], and the iodinated proteins were visualized by autoradiography. Immunostaining with murine MoAb R1.3 (IgG1) or BRIC 111 (IgG1) was carried out as described by Towbin et al.[14] The second antibody for detecting antibody bound to the blot was a preparation of horseradish peroxidase-conjugated rabbit immunoglobulins (Igs) to mouse Igs (DAKO Ltd, High Wycombe, UK).

The assay of β(1-4)galactosyltransferase was by the method of Cartron et al.,[15] with the following modifications: 20 μl of plasma or 350 μg of stromal protein was incubated with the acceptor (p-nitrophenyl-N-acetyl-β-glucosaminide) and UDP-^{14}C-galactose (1×10^5 cpm) for 16 and 40 hours, respectively. The reaction mixtures were separated by chromatography on paper (Whatman DE 81, Whatman Ltd, Maidstone, UK) in ethyl acetate-pyridine-water (10:4:3), vol/vol) for 6 hours. The enzyme activity is expressed as the percentage of incorporation of ^{14}C-galactose.

Results

Reactions of Hb M-Hyde Park RBCs with various lectins, MoAbs, and human antisera

The propositus's (M-Hyde Park) RBCs were not agglutinated by lectins from *D. biflorus*, *S. sclarea*, or *L. cardiaca* and reacted weakly with lectins from *A. hypogaea*, *S. horminum*, and *V. cretica*. The negative reaction with *A. hypogaea*, as reported by Bird et al.,[4] may be due to a difference in the concentration of their preparation. Positive agglutination (+++) was obtained with two lectins specific for N-acetylglucosamine (GlcNAc) (i.e., GS II and *V. hyrcanica*) and with some lectins specific for N-acetylgalactosamine (GalNAc) and/or galactose (Gal) (*H. pomatia*, *M. disciformis*, and *B. purpurea*). Moreover, stronger agglutination (++++) was obtained with *G. soja* and *V. villosa* B_4;[16] both are specific for GalNAc. When the agglutination reactions of Tn red cells to monoclonal anti-Tn reagents and those given by the propositus's RBCs were compared, no mixed-field agglutination of M-Hyde Park RBCs was observed, nor were those titration scores as high as those of the Tn RBCs (Table 1). However, the total score with the propositus's cells and *V. graminea*, *U. europaeus*, anti-I, or -i was consistently higher than that obtained using normal group MM, MN, and NN adult RBCs. The titration score with the propositus's RBCs against human anti-i was also much lower than that with cord cells. No enhancement of agglutination with monoclonal anti-N (sialic acid-dependent and -independent antibodies) or rabbit anti-N sera was detected (data not shown).

The propositus's RBCs did not react with monoclonal anti-A (two examples), -B (three examples), and -A,B (two examples). When tested with human antisera, the propositus's RBCs were positive with 2 of 10 sera containing anti-A, with 1 of 10 sera containing anti-B, and with 3 of 10 sera containing anti-A,B. One of 10 AB serum samples gave a weak positive reaction. The results of testing in South Africa were that the RBCs gave weak positive reactions with some but not all random ABO-compatible human adult sera and not with cord sera. The following low-frequency MNS-related antigens were not detected on his cells: Cl^a, Dantu, He, Mt^a, M^g, M^v, Mit, Ny^a, Ri^a, s^D, St^a, Wr^a, Wb, and the Miltenberger complex of antigens (antisera used: Mi^a, Vw, Mur, Hut, Hil, and Raddon).

The plasma of the propositus did not contain anti-Tk, but anti-T and -Tn were detected.

Table 1. *Titration scores with lectins, monoclonal anti-Tn's, and human antisera*

Reagents	Hyde Park (group NN)	Natal control (group NN)	Group MN	Tn RBCs
V. graminea†	23	11†	11	
U. europaeus	54	39	39	
Anti-I	80	60	NT*	
Anti-i	24	1	(cord cells: 72)	
Anti-Tn				
BRIC 111	33			49
BRIC 113	39			55
BRIC 114	34			58

* Not tested.
† Group MM cells: negative with *V. graminea*

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Sialic acid content and labeling of membrane components of Hb M-Hyde Park RBCs

Titrations of Polybrene and *L. polyphemus* with M-Hyde Park RBCs did not show any significant loss of sialic acid. Moreover, quantitation indicated that these cells had approximately 90 percent of the total sialic acid content of normal RBCs. The fluorograph of tritiated sialoglycoproteins (Fig. 1) showed that almost no radiolabeling of the delta dimer had occurred on the propositus's RBCs. The uptake of tritium by the alpha and the delta monomers was also reduced. Similarly, iodination failed to radiolabel these components to the same extent as the corresponding bands on normal RBCs (Fig. 2). On immunostaining with R1.3 (Fig. 3), membrane components consisting of delta sialoglycoprotein usually produce more intense staining than those consisting solely of alpha component. Although normal RBCs usually show three discrete bands of apparent molecular weights (app MW) of 63,000 (alpha delta), 46,000 (delta dimer) and 26,000 (delta monomer), the propositus's RBCs produced a series of diffuse bands of app MW 58,000 to 63,000 and a broad staining band of app MW 43,000 to 46,000. The latter band corresponds to the region between the delta dimer and the alpha monomer where no radiolabeling occurred (Figs. 1 and 2). The delta monomer stained with the same intensity as the delta component of normal RBCs (Fig. 3).

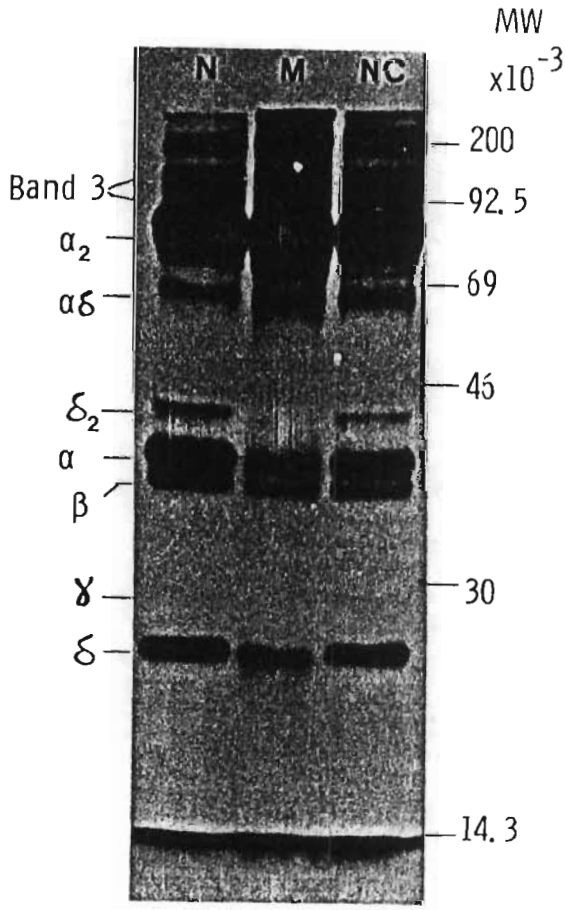

FIG. 1. Tritium labeling of sialoglycoproteins ($NaIO_4/NaB^3H_4$) on normal (N), M-Hyde Park (M), and natal control (NC) red cells.

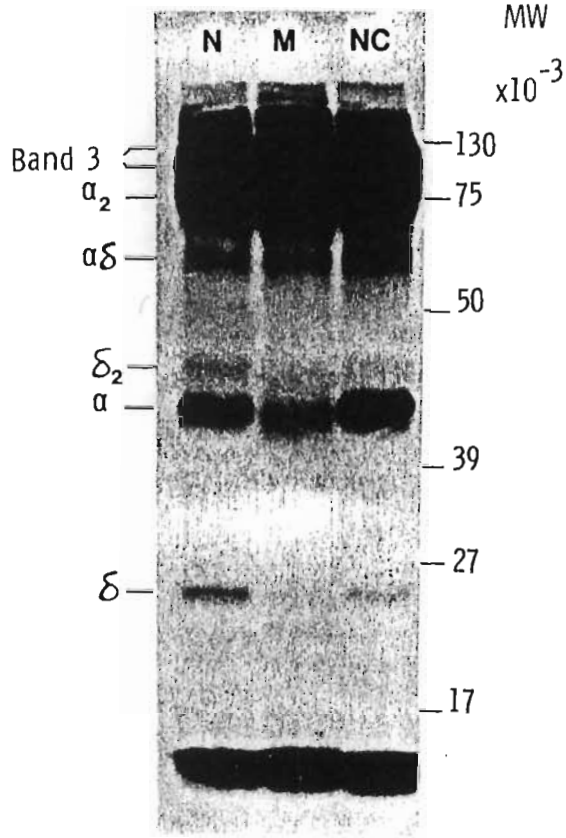

FIG. 2. Iodination of membrane proteins on normal (N), M-Hyde Park (M), and natal control (NC) red cells.

Use of enzymes to distinguish the sites of agglutination detected by lectins

Trypsin, papain, and endo-β-galactosidase were used to distinguish the various membrane components interacting with lectins or antisera. For instance, both the alpha and delta sialoglycoproteins are susceptible to papain treatment, whereas only the alpha sialoglycoprotein is trypsin-sensitive.[17] Endo-β-galactosidase specifically cleaves the internal β(1-4)galactosidic linkage in a polylactosamine-type structure [Galβ(1-4)GlcNAcβ(1-3)].[18] The GlcNAc residues remaining at the terminal position of the oligosaccharide chain react with GS II, and RBCs treated with this enzyme are Tk-activated.[19]

After trypsin treatment the propositus' RBCs were no longer agglutinated by the monoclonal anti-Tn reagents. Trypsinized Tn RBCs showed a slight change in the strength of agglutination with the MoAbs, which was abolished after papain treatment (data not shown). Immunostaining using MoAb BRIC 111 showed that the sialoglycoproteins in M-Hyde Park RBCs were only weakly reactive with the Tn antibody, whereas the Tn-positive RBCs produced stronger and broader staining bands (Fig. 4). The immunostaining

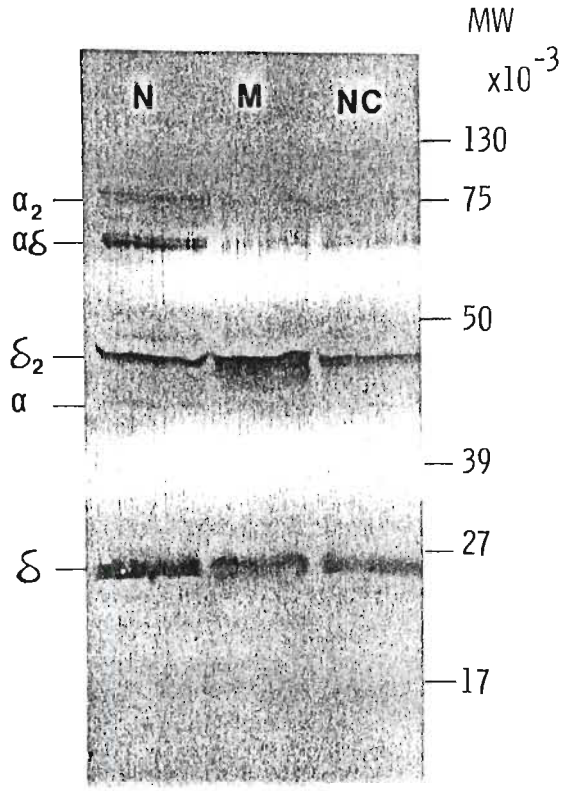

FIG. 3. Immunostaining of alpha and delta sialoglycoproteins using MoAb R1.3 on normal (N), M-Hyde Park (M), and natal control (NC) red cells.

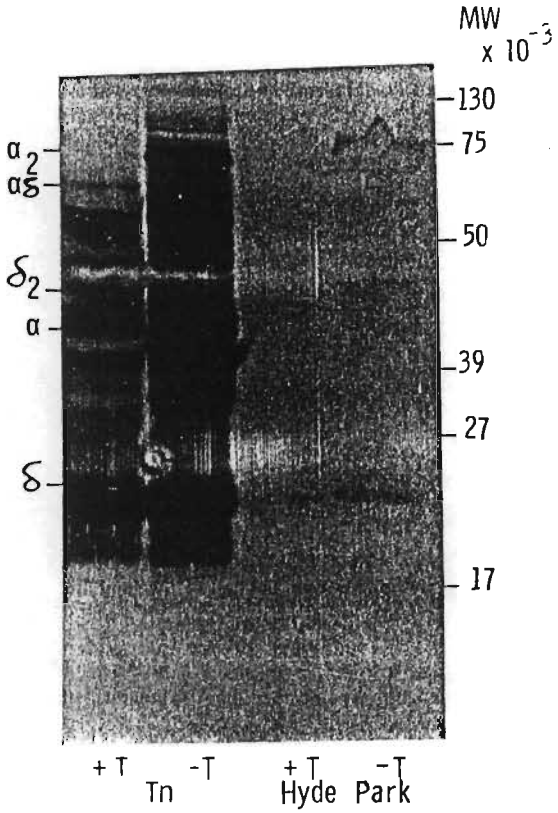

FIG. 4. Immunostaining of Tn red cells (20 μl of stroma) and M-Hyde Park red cells (35 μl of stroma) with MoAb BRIC 111 before and after trypsin (T) treatment.

result was therefore not consistent with the titration scores obtained with monoclonal anti-Tn (Table 1).

The titration score of trypsinized M-Hyde Park RBCs with *V. graminea* was identical to the scores of the trypsinized normal RBCs, irrespective of their MN status (Table 2). All the reactivity of the cells with *V. graminea* was removed after papain treatment. However, treatment of these cells with endo-β-galactosidase (i.e., in vitro Tk activation) did not significantly affect the titration scores of the treated RBCs with *V. graminea* (Table 2).

Untreated M-Hyde Park RBCs reacted with GS II and *V. hyrcanica*. The reaction with these two lectins prior to any enzyme treatment is indicative of exposed terminal GlcNAc residues. After endo-β-galactosidase treatment, M-Hyde Park RBCs produced agglutination reactions (Table 3) identical to those reported for the in vivo Tk-activated RBCs, i.e., reduced H and I activity and enhanced reaction with GS II after protease treatment.[20,21] The localization of these sugar residues was determined by incubating the propositus's RBCs with UDP-^{14}C-galactose and bovine milk β-galactosyltransferase, which only transfers ^{14}C-galactose in (1-4) linkage to terminal GlcNAc residues. Figure 5 shows that ^{14}C-galactose was incorporated into two diffuse regions of app MW of 88,000 to 130,000 and 46,000 to 73,000 in M-Hyde Park RBCs. None of the radioactivity was associated with MN-sialoglycoproteins. On SDS-PAGE, RBC glycoprotein Bands 3 and 4.5 are located at app MW 90,000 to 105,000[22] and 50,000 to 60,000,[23] respectively. These figures are within the molecular weight ranges of the ^{14}C-labeled membrane components. After endo-β-galactosidase treatment, the propositus's RBCs and the in vitro Tk-activated RBCs produced identical radiolabeled patterns (Fig. 5). Further proof of the GlcNAc residues' acting as an acceptor of Gal was the loss of agglutination reactions with GS II and *V. hyrcanica* after incubating these cells with unlabeled UDP-galactose and bovine milk β-galactosyltransferase (data not shown). This enzymatic transfer of Gal onto RBC membrane components did not affect the enhanced agglutination reaction detected with

Table 2. *Titration scores of* V. graminea *before and after enzyme treatment*

Enzyme treatment	Titration scores with *V. graminea*			
	MM	MN	Natal control	Hyde Park
Untreated	0	13	17	28
Treated with trypsin	49	49	48	48
Treated with papain	0	0	0	0
Untreated	0	15	21	28
Treated with endo-β-galactosidase	2	16	26	29

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Table 3. *Effect of endo-β-galactosidase on titration scores with* U. europaeus, *anti-I, -i, GS II, and* V. hyrcanica

Lectin/Antiserum	Treatment	Hyde Park	Natal control
U. europaeus	None	44	33
	Endo-β-galactosidase	36	27
Anti-I	None	48	33
	Endo-β-galactosidase	34	23
Anti-i	None	19	0
	Endo-β-galactosidase	0	0
GS II	None	10	0
	Papain	61	0
	Papain + Endo-β-galactosidase	99	86
V. hyrcanica	None	10	0
	Papain	60	0
	Papain + Endo-β-galactosidase	107	98

V. graminea, nor the reactions with *U. europaeus,* anti-I, and -i.

The presence of terminal GlcNAc residues on M-Hyde Park RBCs implies either incomplete or increased glycosylation. The former was tested by assaying the activity of β(1-4)galactosyltransferase using a synthetic acceptor for ^{14}C-galactose. When stroma or plasma from the propositus was used as the enzyme source, the level of activity was found to be greater than normal (Table 4). However, the plasma from the propositus failed to incorporate any ^{14}C-galactose onto his own RBCs. This may be due to the different specificity of the plasma galactosyltransferase toward natural acceptors on RBCs. Alternatively, this plasma enzyme is not involved in the biosynthetic reaction in vivo.

Discussion

A panel of lectins is usually adequate for identifying cryptantigens involved in polyagglutination.[3] In addition, polyagglutinable RBCs react weakly with many ABO-compatible human sera. The present study describes a sample of RBCs from a healthy individual having Hb M-Hyde Park, the reactions of which with certain lectins would have implied that the blood group N antigen was enhanced and that both Tn (as shown with monoclonal anti-Tn) and Tk were present on the RBCs. Most lectins have binding sites that are predominantly for monosaccharides. Thus, our interpretation of the data in this study is based on the specificity of the monosaccharides and not on the agglutination of a blood group antigen by a lectin. In addition, this study presents evidence that when the agglutination reactions produced by specific lectins and MoAbs are not identical, biochemical and/or immunochemical techniques are required to determine the site(s) of agglutination.

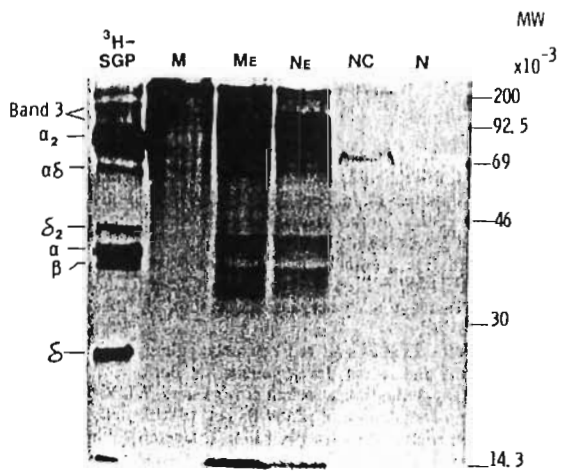

FIG. 5. Enzymatic transfer of ^{14}C-galactose onto normal (N), M-Hyde Park (M), and natal control (NC) red cells. ^{3}H-SGP: tritiated sialoglycoproteins; ME: M-Hyde Park cells after endo-β-galactosidase treatment; NE: normal red cells after endo-β-galactosidase treatment.

Table 4. *β(1-4)galactosyltransferase activity in plasma and stroma*

	Percentage of ^{14}C-galactose incorporated	
Cells	Plasma	Stroma
Hyde Park	85.8	26.3
Natal control	82.8	16.8
Normal donor		
1	72.0	7.2
2	75.7	10.2
3	69.2	10.5
4	81.8	12.1

The propositus's RBCs are not Tn; they do not react with the Tn-specific lectin *S. sclarea*[24] or with *D. biflorus,* even though they are agglutinated by monoclonal anti-Tn reagents and other lectins specific for N-acetylgalactosamine. Immunostaining with monoclonal anti-Tn revealed that the M-Hyde Park RBCs do not possess any reactive Tn sites. The strong agglutination reactions obtained with this and other monoclonal anti-Tn reagents therefore imply crossreactivity (Table 1). The sialic acid content of the RBCs and the titration scores with Polybrene and *L. polyphemus* indicate that there is very little change in the overall negative charge of the RBC surface. However, the result from the tritium labeling of sialic acid (Fig. 1) suggests reduced sialic acid content on the sialoglycoproteins (SGPs). Because these components were also weakly labeled by Iodine-125, the polypeptide moiety of these glycoproteins is likewise abnormal (Fig. 2). Immunostaining of M-Hyde Park RBCs with R1.3 confirmed that the cell membrane abnormality lies not only in the heterogeneity of the sialylation, but also in the variable molecular size of the delta SGP (Fig. 3).

The above findings probably explain the unusual agglutination reactions obtained with the proposi-

tus's RBCs and the anti-N lectins used. His RBCs reacted normally with both monoclonal and rabbit anti-N reagents, indicating that they have normal expression of blood group N antigen. Their enhanced reaction with *V. graminea* may be analogous to the intensified agglutination expected with desialylated MN glycoproteins[25] and neuraminidase-treated red cells.[26,27] Furthermore, *V. graminea* binds more strongly to the clusters of O-linked disaccharides (i.e., Gal β[1-3]GalNAc-O-Ser/Thr) along the first eight amino acid residues of desialylated glycopeptides than to the group of O-linked oligosaccharides located on amino acid residues 10 to 15 of the polypeptide chain.[25] It is therefore possible that heterogeneous sialylation, as detected by weak agglutination with *A. hypogaea* and persistent reduction in the uptake of radiolabeling by the alpha and delta SGPs, is associated with the same region as that recognized by *V. graminea*. Although abolition of the enhanced reaction of the propositus's RBCs with this lectin after trypsin treatment (Table 2) is indicative of a trypsin-sensitive component, the SDS-PAGE analysis shows no evidence of a new band.

The heterogeneity in sialylation of the propositus's SGPs may have concomitantly produced other serologic anomalies, for example, the enhanced reactions with *U. europaeus*, anti-I, and -i. This enhancement is moderate in comparison with that of I or i RBCs treated with neuraminidase.[28] However, the copresence of I and i antigenic activities on these RBCs may also show that the RBCs have not yet reached their adult stage; as occurs in other forms of bone marrow stress caused by anemia, the RBCs may still be relatively undifferentiated.[29]

Besides the SGPs, a second polyagglutination receptor was detected on the propositus's RBCs by GS II and *V. hyrcanica* lectins. Both these lectins are specific for N-acetylglucosamine (GlcNAc).[30,31] Their reactions were not related to the enhanced positive reaction obtained with *V. graminea* (Table 2); therefore, the reaction was not associated with the O-linked oligosaccharide chains. The ^{14}C-galactose incorporation experiment showed that M-Hyde Park RBCs do not have any new sites other than Band 3 and Band 4.5 as acceptors for Gal (Fig. 5). These two glycoproteins and polyglycosylceramides normally carry the H, I, and i blood group antigenic determinants on their N-acetylglucosamine-containing oligosaccharide chains.[29] The exposed terminal GlcNAc residues are therefore associated with the N-linked (i.e., R-GlcNAc-N-Asparagine) polylactosaminoglycan on Band 3 and Band 4.5[32-34] and are not related to the sialoglycoproteins, which also have N-linked chains but of a different type.[35] Terminal GlcNAc on the RBCs of an healthy individual is in itself unusual because it indicates a defect in glycosylation. At present the reason for GlcNAc exposure seems unlikely to be a depressed level of β(1-4)galactosyltransferase, because the enzyme level on the propositus's RBCs and plasma is strong (Table 4).

In conclusion, the polyagglutinability of this sample of M-Hyde Park RBCs is due to two unrelated abnormalities. The first is associated with the heterogeneity in the molecular size of SGPs and a mild reduction in the sialylation of O-linked oligosaccharide chains on these membrane components. The second is caused by the exposure of terminal N-acetylglucosamine on polylactosamine-type, N-linked carbohydrate chains of Band 3 and Band 4.5. As there are separate pathways for the synthesis of O- and N-linked oligosaccharides,[36,37] and as the defects are most probably confined to the nonreducing terminal of these carbohydrate chains, the present findings shed no new light on how a hemoglobin variant can influence both glycosylation processes simultaneously, resulting in abnormal carbohydrate chain expression. A sample of normal RBCs, treated with acetyl phenylhydrazine to induce oxidant damage and subsequently tested with lectins and human sera, showed no signs of polyagglutination. Nor was polyagglutination observed in two individuals who have other types of unstable hemoglobin.

There have been two reports of M-Hyde Park occurring in a large family.[38,39] The present case is the first in which this variant hemoglobin has been found in close association with polyagglutinable RBCs.

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May-Jean King, PhD, Scientific Officer, Biochemical Reference Section, Blood Group Reference Laboratory, Harkness Building, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, England. [Reprint requests]

Yew Wah Liew, BSc, Scientific Officer, Serological Reference Section, Blood Group Reference Laboratory.

Phyllis P. Moores, PhD, Head, Immuno-Haematology Research Laboratory, Natal Institute of Immunology, Durban, South Africa.

George W.G. Bird, DSc, PhD, MBSS, FRCPath, Director, Blood Group Reference Laboratory; Current address: Regional Blood Transfusion Centre, Edgbaston, Birmingham, England.

Paper 45

LECTINS FROM **GRIFFONIA SIMPLICIFOLIA** (GS II) AND **VICIA HYRCANICA** INTERACT WITH TERMINAL N-ACETYL-D-GLUCOSAMINYL RESIDUES ON A SAMPLE OF HUMAN ERYTHROCYTES WITH HB M-HYDE PARK.

M-J. King[1], Y.W. Liew[1], P.P. Moores[2] and G.W.G. Bird[1]

[1]Blood Group Reference Laboratory, Radcliffe Infirmary, Woodstock Road, Oxford, OX2 6HE, England.

[2]Natal Blood Transfusion Service, P.O. Box 2356, Durban 4000, South Africa.

The lectin from **Griffonia simplicifolia** (GS II), formerly **Bandeiraea simplicifolia** II (BS II) is specific for N-acetyl-D-glucosamine (GlcNAc) (1). It agglutinates human red cells carrying the cryptantigen Tk, which is responsible for one form of human erythrocyte polyagglutination. **In vivo** exposure of Tk was reported on individuals with, for example, **Bacteroides fragilis** infection and the agglutination reaction of these Tk-activated red cells with GS II is enhanced by subsequent protease treatment (2). **In vitro** unmasking of GlcNAc residues on red cells (or Tk-activation) occurs after treatment with an endo-β-galactosidase from **E. freundii**(3), **B. fragilis**(4), or **F. keratolyticus** (5). This study presents the agglutination reactions of a sample of Hb M-Hyde Park red cells with GS II and **V. hyrcanica** (6). The membrane components involved were identified by an enzymic radiolabelling technique. For comparison two types of red cells with abnormal glycosylation were used. En(a-) erythrocytes lack the major MN-sialoglycoprotein (synonym: PAS-1 and PAS-2, or glycophorin A dimer and monomer) and have increased glycosylation on the Band 3 glycoprotein (7,8) whereas erythrocytes from patients with congenital dyserythropoietic anaemia type II (HEMPAS) have an apparent reduction in the glycosylation of Band 3 and Band 4.5 (9,10), while polylactosaminyl carbohydrates are accumulated as glycolipids (11,12). HEMPAS red cells belong to another form of polyagglutination in which there is an increase in blood group i antigenic activity.

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Materials and Methods

Hb M-Hyde Park red cells and normal red cells (to be called Control) were obtained from the Natal Blood Transfusion Service (South Africa). Fresh normal red cells were obtained from our laboratory staff. En(a-) red cells (GW) were obtained from Dr. P. Sistonen (Finnish Red Cross Transfusion Service, Finland), and HEMPAS red cells (MB) was kindly provided by Miss E. Lloyd (Royal Postgraduate Medical School, UK). A crude extract of **V. hyrcanica** was prepared by grinding 1 g of seeds (obtained from University of Southampton, UK) with 10ml of phosphate buffered saline (pH 7.2), centrifuged and the supernatant was used. A partly purified endo-β-galactosidase concentrate was obtained by ammonium sulphate precipitation (to 75% saturation) of a culture supernatant of **Bacteroides fragilis** (provided by Dr. D. Rogers, Portsmouth Polytechnic, UK) and chromatography on a column of CM-50 Sephadex (5 x 30cm) (13). The fractions eluted with 0.3 M sodium acetate buffer (pH 5) were pooled and dialysed against distilled water. After lyophilization, the powder was reconstituted to one-tenth volume of the initial culture supernatant.

For endo-β-galactosidase treatment, 100 µl of enzyme concentrate were incubated with 50 µl of packed red cells at 37°C for 3 hours with intermittent shaking. For papain treatment, equal volumes of packed red cells and papain (10 mg/ml, BDH) were incubated at 37°C for 15 minutes. Labelling of terminal GlcNAc with ^{14}C-galactose was carried out by incubating 40 µl of packed red cells with 30 mU of bovine milk β-galactosyltransferase (1 U/ml, Sigma) and UDP-^{14}C-galactose (2 x 10^5 cpm, specific activity 297 mCi/mmol, Amersham) in 80 µl of 50 mM Tris-HCl buffer (pH 7.8) containing 15 mM $MnCl_2$ and 0.15M NaCl at 37°C for 6 hours with intermittent shaking (14). The radiolabelled red cells were washed, and lysed in 10 mM Tris-HCl (pH 7.8). The stroma preparations (40 µl) were solubilised in 40 µl of sample buffer (containing 5% SDS, 25 mM Tris, 5 mM EDTA-Na_4, 10% glycerol, 0.01% bromophenol blue and 10% mercaptoethanol) and boiled at 100°C for 3 minutes. After SDS-PAGE (15), the 10% gel was fixed in methanol/acetic acid/water (30:20:50, v/v/v) for 1 hour, treated with dimethylsulphoxide (DMSO) for 20 minutes and left in 22% PPO in DMSO for 2 hours at room temperature. After washing in distilled water for 10 minutes, the gel was dried. The dried gel was exposed to a preflashed X-ray film (Cronex 4 from DuPont, UK) at -70°C for 10 to 14 days (16). Haemagglutination scores in titrations of GS II (from 100 µg/ml, Sigma) and **V. hyrcanica** were determined according to Marsh (17).

Results

A combination of papain and endo-β-galactosidase was used to treat red cells for a preliminary identification of the probable receptor sites for the lectins GS II and **V. hyrcanica**. Papain treatment of intact red cells removes most of the exterior portions of the sialoglycoproteins (namely glycophorins A,B and C) (18) while the glycosylated domain of Band 3 remains attached to the cell membrane (19). On the other hand Band 4.5 on intact red cells is fairly

resistant to protease treatments (20). Endo-β-galactosidase specifically cleaves the internal galactosyl β (1-4) N-acetylglucosaminyl linkage in the polylactosamine-type carbohydrate chains, which are normally found on Band 3, Band 4.5 and polyglycosylceramides (21). Table 1 shows that En(a-) cells were agglutinated by GS II after endo-β-galactosidase treatment. This strong agglutination may be due to the increased glycosylation of the N-linked oligosaccharide chain on Band 3 of En(a-) cells. On the other hand, the agglutination reactions of endo-β-galactosidase-treated HEMPAS and normal red cells with GS II were detectable only after subsequent treatment with papain. Their titration scores are identical to the En(a-) cells treated under the same conditions. These results show that the GlcNAc residues on En(a-) and HEMPAS cells, like those on normal red cells, can only be detected in agglutination by GS II and **V. hyrcanica** lectins after endo-β-galactosidase treatment. On the contrary, M-Hyde Park red cells produced weak reaction with GS II or **V. hyrcanica** lectin prior to any enzyme treatment, implying an **in vivo** exposure of terminal GlcNAc residues (Table 1). Papain treatment enhanced the agglutination reaction. This indicates that the GlcNAc residues are not located on the glycophorins. Subsequent endo-β-galactosidase treatment of the papainised red cells produced higher agglutination scores. This reaction is therefore analogous to that of **in vitro** Tk-activation on normal red cells.

The localization of free terminal GlcNAc residues was determined by incubating red cells with UDP-^{14}C-galactose and bovine milk β-galactosyltransferase. This enzyme incorporates ^{14}C-galactose in β(1-4) linkage to GlcNAc residues. Native En(a-) cells have some unsubstituted GlcNAc residues on Band 3 as acceptor for ^{14}C-galactose (Fig. 1.) although they were not detectable by agglutination using GSII lectin (Table 1). Low molecular-weight components in HEMPAS cells (15K-16K), presumably glycolipids, were weakly labelled (Fig. 1). After endo-β-galactosidase treatment, Band 3 (88K-100K) on En(a-) cells was more heavily radiolabelled than a new band of apparent molecular weight (MW) of 50K. In addition to the glycolipids at 15K-16K, two new components were labelled on HEMPAS cells at 41.5K and 31K (Fig. 1). Using M Hyde Park red cells, ^{14}C-galactose was incorporated into 2 diffuse regions of apparent MW of 88K to 130K and 46K to 73K (Fig. 2). After endo-β-galactosidase treatment, both the M-Hyde Park red cells and the **in vitro** Tk-activated Control cells produced identical radiolabelled pattern. The masking of GlcNAc residues by galactose was demonstrated by the loss of agglutination reactions of all the test cells with GS II and **V. hyrcanica** after incubation with unlabelled UDP-galactose and bovine milk β-galactosyltransferase (data not shown).

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Table 1

Effects of endo-β-galactosidase and papain on titration scores with GS II and V. hyrcanica

		Titration Scores
Cells	**Treatment**	**GS II**
Normal	None	0
	Endo-β-gal'dase	0
	Endo-β-gal'dase + papain	123
En(a-)	None	0
	Endo-β-gal'dase	65
	Endo-β-gal'dase + papain	123
HEMPAS	None	0
	Endo-β-gal'dase	0
	Endo-β-gal'dase + papain	123

	Treatment	**GS II**	**V.hyrcanica**
Control	None	0	0
	Papain	0	0
	Papain + endo-β-gal'dase	86	98
Hyde Park	None	10	10
	Papain	61	60
	Papain + endo-β-gal'dase	99	107

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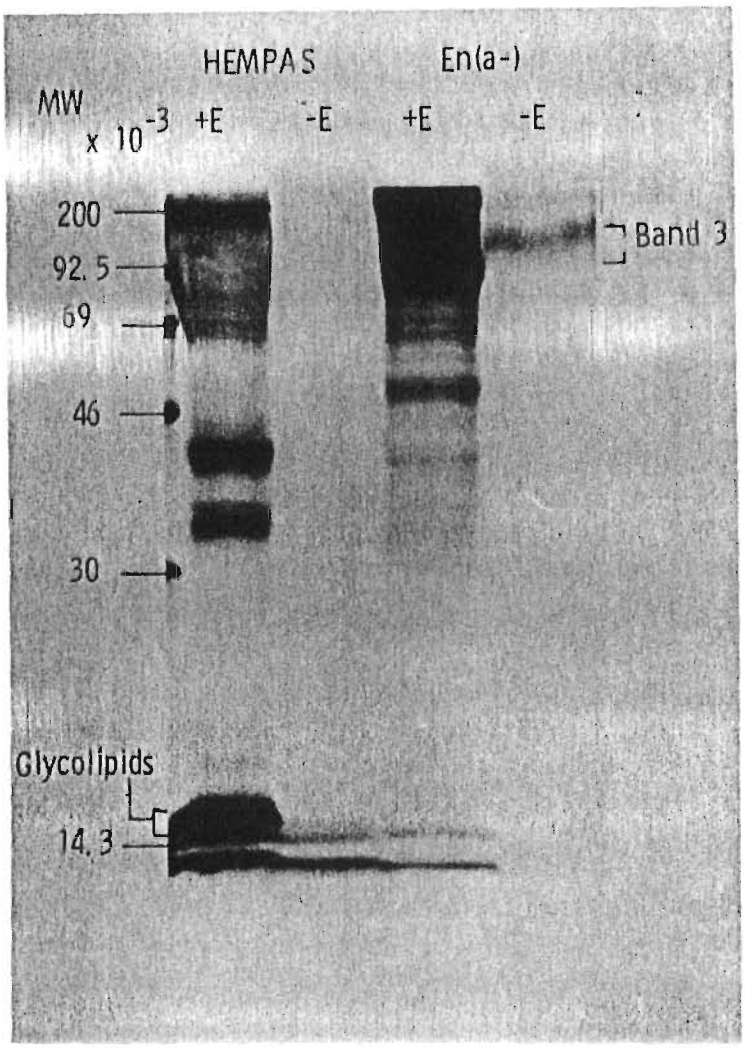

Fig 1. Fluorograph of ^{14}C-galactose incorporation onto En(a-) and HEMPAS red cells before and after endo-β-galactosidase (E) treatment.

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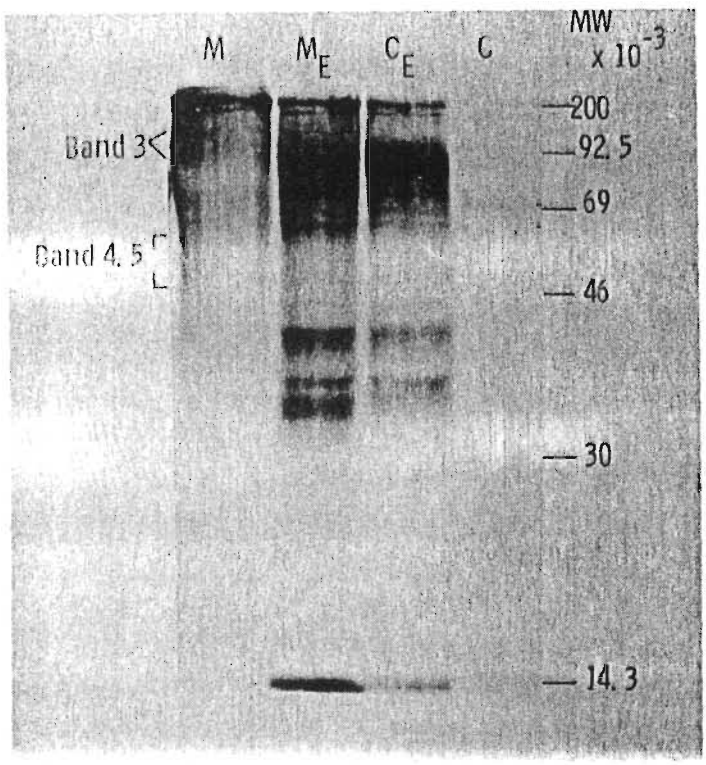

Fig 2. Fluorograph of ^{14}C-galactose incorporation onto M-Hyde Park red cells (M) and normal red cells from Natal (C) before and after endo-β-galactosidase (E) treatment.

Discussion

This sample of Hb M-Hyde park red cells was obtained from an individual of mixed ethnic origin in a large kindred (22) and its reaction patterns were compared with red cells from a normal individual also from South Africa. Thirteen (5 males and 8 females) of the 28 family members tested were found to have an Hb M variant called Hyde Park, and their red cells showed polyagglutination with various lectins and human sera. The affected family members show mild cyanosis of lips and fingernails but none shows overt clinical abnormalities. In recent examination, the propositus' spleen enlarged to 4cm below the costal margin. Although two reports have been published on M-Hyde Park occurring in a large family (23, 24, 25), this case is the first report of polyagglutinable red cells appearing in close association with a variant haemoglobin.

The propositus' red cells were polyagglutinable in that the cells reacted with lectins specific for GlcNAc as well as for N-acetylgalactosamine and/or galactose (26). The latter finding implies abnormal glycosylation on the sialoglycoproteins (data not shown). This report only describes the use of different tools available for identifying the abnormality associated with **in vivo** exposure of GlcNAc residues: agglutination using specific lectins and enzymic incorporation of ^{14}C-galactose to label erythrocyte membrane components carrying this sugar. Although the GS II lectin is normally used in blood group serology to confirm red cell polyagglutination due to exposure of cryptantigen Tk, the reaction of this lectin with native M-Hyde Park red cells is not indicative of the appearance of Tk antigen, which is illustrated in Figure 2. Moreover the defect is not the same as those found on En(a-) cells or HEMPAS red cells (Fig. 1).

At present no explanation can be offered for the **in vivo** appearance of GlcNAc residues of an apparently healthy donor. It remains to be seen as whether haematological abnormality will develop in the affected individuals. It is possible that the red cells from these individuals may not have reached their mature form as some of their peripheral blood samples showed mild macrocytosis and reticulocytosis (22).

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CHAPTER X

MONOCLONAL ANTIBODIES

X.1 PAPERS

Report on 30 monoclonal anti-A fluids studied for the First International Workshop on Monoclonal Antibodies for Human Blood Group and Related Antigens, Paris, 1987
Paper 41 by Moores and Smart

MAbs 1990 workshop studies with monoclonal ABO, Lewis and anti-I.
Paper 49 and part 1 of full report by Moores, Smart and Green Second International Workshop on Monoclonal Antibodies for Human Blood Group and Related Antigens,.1990

MAbs 1990 workshop studies with monoclonal anti-M, anti-N, anti-Lu, anti-RBC(CD44), anti-In(Lu), anti-K2 and anti-Jk^b
Paper 50 and part 2 of full report by Moores, Smart and Green Second International Workshop on Monoclonal Antibodies for Human Blood Group and Related Antigens, 1990

MAbs 1990 workshop studies with monoclonal anti-Rh
Paper 51 and part 3 of full report by Moores, Smart and Green Second International Workshop on Monoclonal Antibodies for Human Blood Group and Related Antigens, 1990

X.2 INTRODUCTION

Monoclonal antibodies (MAbs) for human blood grouping are rapidly gaining in popularity. Briefly, the principle of preparing them with mice is as follows. First, the mouse is immunised with red cells carrying the antigen for which the MAb is required. The mouse's lymphocytes are harvested and, using polyethylene glycol or Sendai virus as the tool, are fused with human myeloma cells. The mouse/human hybridoma cells are now cultured and the clones producing the required MAb selected. The clones are then either cultured or injected into another mouse where they cause a tumour to develop. The MAb is either in the culture medium or the ascitic fluid which is secreted by the mouse's tissues in response to the tumour [Issitt, 1985, p14-15]. Anti-D MAbs are prepared from human lymphoblastoid cell clones infected with Epstein-Barr virus [Issitt, 1985, p648].

The aim of the first international workshop was to study the

activity of MAbs with similar specificities in parallel and compare their results with those of similar polyclonal antibodies. The MAbs for this work were donated by many laboratories and individuals, and were distributed to the workshop participants in their own countries. The participants were encouraged to test them by their own techniques and in accordance with their own conditions, and to include red cells with both common and rare phenotypes from local donors. The results were to be returned to the organisers for analysis.

In the second international workshop, the organisers wanted to compare the activity of MAbs with similar specificities by prescribed techniques and under prescribed conditions using red cells with prescribed phenotypes. The results would be analysed both by computer and manually. The aim was to provide a guide for standardizing MAbs and ensure that their specificities had been defined correctly.

X.3 COMMENTARY

X.3.1 Attainments in Durban, 1961 to 1991

X.3.1.1 First International Workshop, Paris, 1987

The authors received 30 samples of different anti-A MAbs and were asked to do "all you can" to test them [paper 41]. In addition to A_1, A_2, A_1B, A_2B, B and O, they decided to use A_{bantu} and $A_{bantu}B$ red cells as, although rare elsewhere, these phenotypes were readily available locally. The A antigen of the A_{bantu} and $A_{bantu}B$ somewhat resembled that of A_x red cells. The following techniques were selected for use in test tubes: titration, avidity, two-stage enzyme, emergency spin with red cells suspended in saline, emergency spin with red cells suspended in LISS (low ionic strength solution), inhibition by various substances, inactivation and absorption-elution. The MAbs were also tested by automation and microwell techniques, the temperature of the tests and pH of the saline was varied and red cells of other phenotypes and those of animals were included.

Initially, the authors had considerable difficulty reading the

results of their tests, as the red cells stuck firmly to the test tube glass. They overcame this problem by immersing the test tubes before use in a 1% gelatin solution, then rinsing and drying them. The ordinary test tube washing procedure subsequently removed the gelatin adequately. This adaptation avoided possibly over-diluting the MAbs by adding costly bovine albumin, or by adding AB serum which might inhibit them. In the titrations, unlike polyclonal anti-A reagents, many MAbs failed to distinguish by titre or score between A_1 and A_2 or between A_1B and A_2B red cells. Surprisingly, most agglutinated the $A_{bantu}B$ red cells strongly and the A_{bantu} red cells either weakly or not at all. Some seemed merely to have anti-A_1 specificity. In the avidity tests, while reacting well with A_1, A_2, A_1B and A_2B red cells, the majority of the MAbs failed to agglutinate the A_{bantu} and $A_{bantu}B$ red cells. By two-stage enzyme technique, the titres with the A_{bantu} red cells were significantly increased. The authors confirmed this with further examples of A_{bantu} red cells. Variable results were obtained by emergency spin technique, and only minimal improvement was seen when the red cells were suspended in LISS. In the absorption-elution studies, the eluates were made by the 56°C technique of Landsteiner and Miller [1925]. Those from the A_{bantu} were found to react more strongly than those from the A_2 red cells. As the eluates distinguished clearly between A_1 and A_2 and between A_1B and A_2B red cells, their activity more closely resembled that of polyclonal than monoclonal anti-A. Minor alterations in activity were detected when the temperature and pH were changed. Some but not all the MAbs were inhibited by N-acetyl-D-galactosamine but none by D-galactose, α-L-fucose or N-acetyl-D-glucosamine. Most of the MAbs were inhibited by pooled A secretor saliva, and some were even inhibited by pooled non-secretor saliva. The results of the automation tests were disappointing, but those made by microwell technique were excellent. One MAb was contaminated with anti-Le^a and another with antibodies of unknown specificity.

X.3.1.2 Second International Workshop, Lund, 1990

The authors received samples of 96 MAbs with specificities for antigens, not only in the ABO, but also in the MNSs, Rh, Lutheran, Kell, Lewis and Kidd blood group systems and for other antigens. They were asked to test the ABO, MN and anti-D MAbs by specified techniques and with red cells of specified phenotypes.

X.3.1.2.1 ABO system MAbs

The techniques specified for the anti-A, anti-B and anti-A+B MAbs were adhered-to strictly, and red cells with the phenotypes specified were used [paper 49 and part 1 of full report]. A_{bantu}, $A_{bantu}B$ and other cells with weak ABO phenotypes were also included for interest. In the titrations, all except one of the 13 anti-A MAbs reacted to equal titres with A_1, A_2, A_1B and A_2B red cells. In general, their quality seemed much improved, compared with those which the authors had tested in the first workshop. Most of the anti-A MAbs had excellent avidity. The specificity of one MAb was confirmed as anti-A_1, and another gave anti-A_1-like results. Two of the anti-A MAbs, despite giving titres of 512 with red cells of common phenotypes, failed to agglutinate A_{bantu} red cells. All 18 of the anti-B MAbs gave good, avid and specific results. The anti-"acquired" B MAb could not be tested as the necessary control cells were not available. All five anti-A+B MAbs gave good, avid and specific results, but only two agglutinated the A_{bantu} red cells. In the avidity tests, only one anti-A+B MAb reacted with both the A_{bantu} and $A_{bantu}B$ red cells.

X.3.1.2.2 Lewis system MAbs

All three anti-Le^a MAbs gave specific results and appeared suitable for use as reagents by one-stage enzyme technique. Three of the four anti-Le^b MAbs also reacted specifically; again, the one-stage enzyme was the technique of choice.

X.3.1.2.3 Anti-I MAbs

Only one of the two anti-I MAbs gave specific results. The other agglutinated all except O_h red cells. Its specificity was not anti-H, however, as group A_1, A_2, B and O red cells were agglutinated from strongly to weakly in the order $A_1 \rightarrow A_2 \rightarrow B \rightarrow O$ (the group O red cells were the weakest, not the strongest, agglutinated).

X.3.1.2.4 MNSs system MAbs

The techniques specified and the red cells with specific phenotypes requested were employed with care and attention to detail [paper 50 and part 2 of full report]. In the titrations, three of the five anti-M MAbs detected *M* dosage. Three agglutinated MS+ more strongly than MS- red cells. Some reacted best by tile technique, and all were sensitive to pH changes. The results by immuno-blotting technique confirmed that all five were specific for M. Among the seven anti-N MAbs, only one was specific for N. As expected, it was sensitive to pH changes. Their results by immuno-blotting technique were difficult to understand.

X.3.1.2.5 Lutheran system MAbs

Three MAbs for high frequency antigens in the Lutheran system were received. Two were confirmed specific. The specificity of the third was not established with certainty.

X.3.1.2.6 Kell system MAbs

The single example of anti-K2 gave specific results. The authors found that it worked best by one-stage enzyme technique at 37°C.

X.3.1.2.7 Kidd system MAbs

Although the titre with the single example of anti-Jk^b was low, this MAb gave excellent results. The authors found that it

worked well by saline technique at room temperature.

X.3.1.2.8 Rh system MAbs

Four IgM and 22 IgG anti-D, one anti-c, one anti-E, two anti-e and one anti-e-like MAbs were received. The techniques specified for testing the anti-D MAbs were used, together with the red cells of specified phenotypes [paper 51 and part 3 of full report]. Dr P. Tippett (London, UK) kindly graded two local D^u red cell samples for comparison with others submitted by the workshop participants. The anti-c, anti-E and anti-e MAbs were all tested by the authors' techniques.

X.3.1.2.8.1 Anti-D MAbs

All four type IgM anti-D MAbs were found to be specific. The titres given by three with R_1r cells were moderate, and those of the fourth high. All four MAbs agglutinated three local R_o^d and two local R_o^{dg} red cell samples.

All 22 type IgG anti-D MAbs were shown to be specific for D by indirect antiglobulin technique (IAT): none had anti-Rh^D (mosaic D, category III) specificity. All except six agglutinated the local D^u red cells. By one-stage enzyme technique, only eight of the ten MAbs tested had anti-D specificity, one was inactive, one agglutinated type rr (dce/dce) red cells and none agglutinated the D^u red cells. By two-stage enzyme technique, all the anti-D MAbs gave better results. The D^u cells, however, were again not agglutinated. In the titrations, the titres of the MAbs by IAT ranged from 4 to 512; ten MAbs attained the latter figure. The titres by one-stage enzyme technique were all lower. In the avidity tests, two MAbs gave remarkably strong results and three gave good results with the local R_o^d and R_o^{dg} red cells. By solid-phase technique, 24 of the 26 MAbs tested were found suitable for use as anti-D reagents. The low grade D^u red cells included were agglutinated somewhat better by the type IgG than the type IgM MAbs.

X.3.1.2.8.2 Anti-c, anti-E, anti-e and anti-e-like MAbs

The single anti-c MAb gave splendid results; in the authors' hands, it worked best by IAT. The single anti-E MAb also worked well, by saline technique at room temperature: evidently, it preferred a slightly alkaline medium. One of the two anti-e MAbs gave specific results; the other, which reacted more like anti-Ce, did not have anti-Rh34 specificity. The results with the single anti-e-like MAb confirmed its resemblance to anti-e.

Paper 41

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Report on 30 monoclonal anti-A fluids studied for the First international workshop

Paris, 1987

by P. Moores and E. Smart

Natal Blood Transfusion Service,
Durban, South Africa

INTRODUCTION

Thirty monoclonal anti-A fluids (MAbs) were received from Paris in excellent condition. We decided to test them by as many techniques as possible against A_1, A_2, A_1B, A_2B, B, O, A_{bantu} [1], $A_{bandu}B$ [1] and other potentially informative red blood cell samples. A_{bantu} is a subtype of A, similar to A_x, readily available in our area.

MATERIALS AND METHODS

The blood samples were drawn into CPD anticoagulant. The volume required for use each day was obtained from them under sterile conditions. The red cells were washed three times with saline and 2-5 % suspensions (10 % for the slide tests) prepared in saline. The cell suspensions were monitored daily with peanut anti-T and *Glycine soja* lectins for possible bacterial contamination. The blood samples were also replaced at frequent intervals to avoid deterioration due to age.

We subdivised the MAbs into aliquots and stored them at - 30°C. For the tests, the ascitic, but not the supernatant, MAbs were diluted initially 1/10 with saline to conserve the supply. We prepare our saline fresh each day with our own distilled water. The tests were done either in glass test tubes or on glass microscope slides. The drops of MAbs and red cell suspensions were delivered by means of fine-bore glass Pasteur pipettes which were rinsed three times in tap water followed by three times in saline after each delivery. The incubation times were adhered to strictly and all the samples were tested by one technique before proceeding to the next. Unless otherwise indicated, the tube tests were incubated for 1 hour, centrifuged lightly and read both macro- and micro-scopically. In the titrations, the dilutions

were made with saline. The avidity of the MAbs was assessed by the standard technique. The cell suspensions in LISS were prepared as described by Issitt [2]. The bromelin-treated cells were prepared by mixing four volumes of washed, packed cells with one volume of 0,5 % bromelin solution, leaving the mixture at room temperature for 10 minutes and re-washing the cells three times. The bromelin and LISS tests were incubated for 15 minutes before being read. An IgG anti-D reagent and AB serum were included as controls. The tests by automation technique were made with a *Technicon* auto-analyser. The immediate spin, emergency tests were timed by stop watch. The microwell plates were pre-treated with 1 % gelatin solution before use and the tests read uncentrifuged. In the inhibition tests, the substances were allowed to act for 30 minutes (15 minutes for DTT) before adding the cells. Inactivation at 56°C was for 30 minutes and at 70°C for 10 minutes. The absorption-elution studies were made using the 56°C heat elution technique. The other cells used were type A_{in}, A_{el}, genetically A_2B, genetically $A_{bantu}B$ (cells grouped as « B » with human polyclonal reagents but anti-A_1 only detected in the serum), cord A, A_1I-, Dantu+, O_h, O *Glycine soja*+, O T+, O Tn, rabbit, rat, baboon, sheep, *Vervet* monkey and Guineapig. Further details of all these techniques are available on request.

Initially, we experienced difficulties with reading the results of our tests. The cause was traced to the sensitised red cells having adhered to the test tube glass. We overcame this problem by pre-treating the test tubes with 1 % gelatin solution. The test tubes were filled with this solution, rinsed three times with distilled water and dried at 37°C. The normal washing-up process later removed the gelatin adequately. Other advantages noted were that [1] the MAbs were not diluted further and [2] adding AB serum or bovine albumin to the tests was not necessary.

RESULTS

Titrations

Table I shows that 14 murine, one human and 7 ascitic MAbs failed to react more strongly with the A_1 than A_2 and with the A_1B than A_2B cells. This differed from human polyclonal anti-A. The titres with the $A_{bantu}B$ cells were surprisingly high and yet only 7 murine, one human and 3 ascitic MAbs agglutinated the $A_{bantu}B$ cells, Mab 26 W 14 agglutinated the A_{bantu} and not the $A_{bantu}B$ cells and 34 W 1 showed by reacting with the B and O control cells that it contained other antibodies. MAb 19 W 7 was judged to have given the best results by this technique. MAbs, 6 W 2, 26 W 13 and 18 W 3 all appeared to have anti-A_1 specificity.

Avidity tests

At the initial 1/10 dilution, the ascitic MAbs, 14 W 1, 14 W 2, 19 W 7 and 24 W 1 agglutinated the A_2B cells in 2-3 seconds *(Table II)*. MAb 15 W 4 reacted more slowly at 17 and 15 W 3 at 30 seconds and 18 W 3 failed to react at all. The human MAbs 23 W 1 and 26 W 14 agglutinated the A_2B cells in 7-8 seconds, and all the murine MAbs, except 7 W 3, 28 W 1 and 34 W 1, reacted positively with these cells in from 2-11 seconds. Variable times, ranging from 0-68 seconds, were given by the murine MAbs with the $A_{bantu}B$ cells. Neither the 3 human nor the 2 ascitic

TABLE I

Anti-A MAb titrations. Red cells suspended in saline. Titration end-points determined microscopically.

	Red cell phenotypes							
	A_1	A_2	A_1B	A_2B	A_{bantu}	$A_{bantu}B$	B	O
Murine supernatants								
1 W 3	256	128	256	128	16	64	0	0
5 W 8	1024	1024	1024	1024	8	64	0	0
6 W 2	1024	0	512	0	0	0	0	0
7 W 2	2048	2048	2048	2048	0	32	0	0
7 W 3	32	32	32	64	0	16	0	0
9 W 1	8192	8192	8192	8192	128	2048	0	0
9 W 2	64	64	64	64	1	4	0	0
11 W 3	4096	4096	4096	2048	2	512	0	0
16 W 1	512	128	256	256	0	32	0	0
17 W 1	1024	1024	1024	1024	16	256	0	0
19 W 8	128	128	128	128	16	128	0	0
19 W 10	1024	512	1024	512	32	256	0	0
26 W 1	2048	2048	2048	2048	0	512	0	0
26 W 2	2048	512	2048	512	0	512	0	0
28 W 1	128	128	64	128	0	1	0	0
28 W 2	128	128	128	128	1	16	0	0
31 W 1	4096	4096	8192	4096	32	1024	0	0
34 W 1	32	64	32	32	1	8	1	1
37 W 1	256	128	256	128	0	1	0	0
Human supernatants								
23 W 1	256	256	256	256	0	0	0	0
26 W 13	8	0	4	0	0	0	0	0
26 W 14	512	128	512	256	4	0	0	0
Ascitic fluids								
14 W 1	81920	81920	163480	81920	160	20480	0	0
14 W 2	5120	5120	5120	2560	0	160	0	0
15 W 3	20480	10240	10240	10240	0	160	0	0
15 W 4	10240	640	5120	2560	0	0	0	0
18 W 3	40	0	20	0	0	0	0	0
19 W 7	81920	40960	81920	40960	640	10240	0	0
24 W 1	40960	20480	81920	40960	640	10240	0	0
25 W 3	20480	10240	20480	10240	0	1280	0	0

MAbs 15 W 4 and 18 W 3 agglutinated these cells. MAbs 14 W 1, 19 W 7 and 24 W 1 were the only fluids of ascitic origin which agglutinated the A_{bantu} cells (22-31 seconds), and none of the human MAbs reacted with them. Of the 8 murine MAbs that agglutinated the A_{bantu} cells, 17 W 1, 19 W 8 and 19 W 10 did so the fastest (in 16 to 18 seconds). At 2 minutes, the strongest reactions with the $A_{bantu}B$ cells were given by MAbs 1 W 3, 9 W 1, 17 W 1 and 19 W 7. Of the MAbs with anti-A_1 specificity, 6 W 2 was by far the strongest, agglutinating the A_1 and A_1B cells in 3-4 seconds. The following MAbs, diluted 1/16 (ascitic MAbs 1/160), reacted with the $A_{bantu}B$ cells in 15-29 seconds: 9 W 1, 11 W 3, 14 W 1, 17 W 1, 19 W 7, 19 W 10 and 24 W 1. They were judged to have given the best results by this technique.

TABLE II

Anti-A MAb avidity tests. Red cells suspended in saline.

	Red cell phenotypes							
	A_1	A_2	A_1B	A_2B	A_{bantu}	$A_{bantu}B$	B	O
	Time of first appearance of agglutination in seconds							
Murine supernatants								
1 W 3	2	4	3	4	80	6	0	0
5 W 8	5	5	4	7	28	12	0	0
6 W 2	3		4				0	0
7 W 2	4	6	4	6	0	26	0	0
7 W 3	7	14	12	17	0	0	0	0
9 W 1	3	3	3	3	26	5	0	0
9 W 2	5	9	6	11	0	68	0	0
11 W 3	2	3	2	4	70	7	0	0
16 W 1	3	5	4	5	0	10	0	0
17 W 1	2	2	2	3	16	3	0	0
19 W 8	3	4	3	4	18	9	0	0
19 W 10	3	4	3	4	17	5	0	0
26 W 1	2	3	2	2	0	6	0	0
26 W 2	2	3	2	4	0	5	0	0
28 W 1	26	40	31	42	0	0	0	0
28 W 2	6	8	6	9	0	0	0	0
31 W 1	2	3	4	3	6	4	0	0
34 W 1	10	21	10	18	0	0	0	0
37 W 1	5	5	6	6	0	0	0	0
Human supernatants								
23 W 1	5	8	5	7	0	0	0	0
26 W 1	12	0	0	0	0	0	0	0
26 W 14	6	7	7	8	0	0	0	0
Ascitic fluids								
14 W 1	1	3	2	3	31	4	0	0
14 W 2	1	3	2	2	0	10	0	0
15 W 3	4	16	7	30	0	110	0	0
15 W 4	3	14	6	17	0	0	0	0
18 W 3	0	0	0	0	0	0	0	0
19 W 7	1	3	2	2	25	4	0	0
24 W 1	1	2	2	2	22	4	0	0
25 W 3	3	4	3	4	0	31	0	0

Effect of temperature and pH

Only minor changes in titre and avidity were seen when the MAbs were titrated in parallel at 4, 10, 22 and 37°C. Little variation was observed also when they were titrated in parallel in, and tested with cells suspended in, saline at pH 5.3, 5.6, 6.2, 6.6 and 7.0.

TABLE III

Anti-A MAb titrations. Bromelin treated cells suspended in saline. Titration end-points determined microscopically.

	Red cell phenotypes						
	A_1	A_2	A_1B	A_2B	A_{bantu}	$A_{bantu}B$	B
Murine supernatants							
1 W 3	4096	2048	2048	1024	256	1024	0
5 W 8	1024	1024	1024	1024	128	256	0
6 W 2	4096		1024		0	0	0
7 W 2	1024	1024	1024	512	8	256	0
7 W 3	1024	1024	512	512	128	32	0
9 W 1	8192	8192	8192	4096	8192	4096	0
9 W 2	512	256	512	512	4	32	0
11 W 3	4096	4096	4096	4096	1024	2048	0
16 W 1	2048	1024	2048	1024	4	128	0
17 W 1	4096	2048	4096	2048	4096	8192	0
19 W 8	1024	512	512	1024	128	512	0
19 W 10	2048	2048	2048	2048	512	4096	0
26 W 1	4096	4096	4096	2048	64	4096	0
26 W 2	4096	2048	4096	4096	128	2048	0
28 W 1	1024	512	1024	512	64	0	0
28 W 2	512	512	512	256	64	64	0
31 W 1	8192	4096	4096	4096	131072	262144	0
34 W 1	8192	4096	2048	2048	64	512	4
37 W 1	1024	1024	512	128	64	0	0
Human supernatants							
23 W 1	256	128	256	128	4	1	0
26 W 13	64	0	64	2	0	0	0
26 W 14	1024	512	1024	512	512	0	0
Ascitic fluids							
14 W 1	81920	40960	40960	40960	40960	40960	0
14 W 2	5120	2560	5120	2560	80	160	0
15 W 3	81920	40960	81920	40960	2560	80	0
15 W 4	10240	2560	10240	2560	640	1280	0
18 W 3	80	10	80	10	640	1280	0
19 W 7	327680	163840	327680	163840	2621440	2621440	0
24 W 1	40960	40960	40960	40960	10240	40960	0
25 W 3	20480	20480	20480	20480	320	2560	0

Tests in low ionic strength medium

With cells suspended in LISS rather than in saline, only the results with MAbs 9 W 1 and 9 W 2 were enhanced in strength.

Two-stage bromelin technique

In tests with bromelin-treated A_1, A_2, A_1B and A_2B cells, *Table III* shows that the titres of MAbs 1 W 3, 7 W 3, 9 W 2, 16 W 1, 19 W 7, 19 W 8, 26 W 13, 28 W 1, 34 W 1 and 37 W 1 were significantly higher than when untreated cells were used. The remaining MAbs showed little change. Almost all the MAbs, in particular 31 W 1, also gave higher titres with the $A_{bantu}B$ cells. The most dramatically increased

results, however, were those with the bromelin-treated A_{bantu} cells. Whereas 15/30 MAbs agglutinated these cells untreated, 28/30 now did so. MAbs 7 W 3, 15 W 3, 23 W 1, 26 W 14, 28 W 1 and 37 W 1 even agglutinated the A_{bantu} more strongly than the $A_{bantu}B$ cells! MAbs 15 W 4 and 18 W 3 prozoned with both these cell phenotypes. The results with further A_{bantu} and $A_{bantu}B$ cells, tested in parallel untreated and bromelin-treated, are shown in *Table IV*.

TABLE IV

Anti-A MAbs tested in parallel by saline technique with additional untreated and bromelin-treated A_{bantu} and $A_{bantu}B$ red cells.

Reading scale: 4, 3, 2, 1, ±, (3), (2), (1), (±), 5
macroscopic microscopic

Red cells

	Untreated											Bromelin-treated										
	A_{bantu}							$A_{bantu}B$			B	A_{bantu}							$A_{bantu}B$			B
Samples:	a	b	c	d	e	f	g	h	i	j	k	a	b	c	d	e	f	g	h	i	j	k
Murine supernatants																						
1 W 3	2	3	1	2	3	2	4	4	4	3	-	2	4	3	3	3	4	4	4	4	4	-
5 W 8	1	2	1	1	1	2	2	3	3	1	-	4	4	3	3	4	4	4	4	4	3	-
7 W 2	-	-	-	-	-	-	-	1	1	-	-	(2)	4	(3)	(2)	(3)	(3)	2	1	1	(2)	-
7 W 3	-	(1)	-	(2)	-	-	(1)	1	(3)	-	-	3	3	3	3	3	4	4	1	1	1	-
9 W 1	1	4	2	2	2	2	4	4	4	2	-	4	4	4	4	4	4	4	4	4	4	-
9 W 2	-	-	-	-	-	-	-	1	1	-	-	1	4	3	3	2	3	4	4	2	2	-
11 W 3	-	1	-	-	-	(1)	2	2	2	(1)	-	2	4	3	4	3	3	4	4	4	1	-
16 W 1	-	-	-	-	-	-	-	1	(3)	-	-	(2)	1	(3)	(2)	(1)	(2)	2	3	3	1	-
17 W 1	1	3	1	1	1	2	3	4	4	3	-	3	4	3	3	3	3	4	4	4	3	-
19 W 8	1	3	2	1	2	2	3	3	3	1	-	3	4	3	4	3	3	4	4	4	3	-
19 W 10	1	2	1	(3)	1	1	3	3	3	1	-	3	4	4	4	4	3	4	4	4	3	-
26 W 1	-	-	-	-	-	-	(2)	1	1	(2)	-	1	2	1	1	1	1	3	3	3	3	-
26 W 2	(1)	(2)	-	-	-	-	1	3	3	1	-	2	2	1	1	1	1	3	4	4	4	-
28 W 1	-	-	-	-	-	-	2	-	(±)	-	-	3	4	2	2	2	2	3	1	(2)	-	-
28 W 2	-	1	-	(±)	-	-	1	2	(3)	-	-	2	4	3	3	3	3	4	1	1	-	-
31 W 1	2	4	3	2	3	3	4	4	4	4	-	4	4	4	4	4	4	4	4	4	4	-
34 W 1	1	(3)	-	(1)	1	(±)	-	4	4	3	(±)	3	4	4	4	3	4	4	4	4	4	3
37 W 1	-	-	-	-	-	-	-	-	-	-	-	1	2	1	1	2	2	3	1	(3)	(2)	(±)
Human supernatants																						
23 W 1	-	-	-	-	-	-	-	-	(1)	-	-	-	(1)	(1)	-	(2)	2	3	(2)	(3)	-	-
26 W 14	-	(3)	(±)	(1)	(1)	-	1	-	-	-	-	3	3	2	3	3	2	3	(2)	(2)	-	-
Ascitic fluids																						
14 W 1	2	3	2	3	4	4	4	4	4	2	-	3	4	4	4	4	4	4	4	4	4	-
14 W 2	-	(1)	-	-	-	-	(3)	(3)	1	-	-	1	3	1	2	1	2	4	4	3	1	-
15 W 3	-	-	-	-	-	-	(2)	(3)	(3)	-	-	1	3	1	1	2	1	4	3	3	1	-
15 W 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(3)	-	-	(2)	(3)	-	-	-
19 W 7	3	4	2	2	1	1	4	4	4	4	(1)	4	4	4	4	4	3	4	4	4	4	(3)
24 W 1	2	3	2	1	2	1	3	4	4	4	-	4	4	4	4	4	4	4	4	4	4	-
25 W 3	-	(2)	-	-	-	(1)	1	2	3	2	-	2	3	3	3	3	3	4	4	4	3	(3)

Automation technique

At the dilutions selected (supernatants neat to 1/150; ascitic fluids 1/100 to 1/1000), the majority of the MAbs did not distinguish between the A_1, A_2 and A_1B cells. The 6 A_2B cell samples gave variable strength positive results, and some MAbs agglutinated the 7 A_{bantu} cell samples weakly, others not. In general, the results were disappointing and it is possible that some MAbs were used too dilute.

Immediate-spin technique, cell suspensions in saline

MAb 19 W 7, diluted 1/40, still agglutinated both the A_{bantu} and $A_{bantu}B$ cells. MAbs 5 W 8, 9 W 1, 14 W 1, 17 W 1, 19 W 10 and 24 W 1, when diluted, reacted with all except the A_{bantu} cells. Surprisingly, MAbs 28 W 2 and 31 W 1 agglutinated the A_{bantu} but not the $A_{bantu}B$ cells. The remainder failed to agglutinate the A_{bantu} or both the A_{bantu} and $A_{bantu}B$ cells. MAbs 6 W 2, 26 W 13 and 15 W 4 agglutinated the A_1 and A_1B cells only and 18 W 3 the A_1 cells only.

Immediate-spin technique, cell suspensions in LISS

The results with 9 W 1, 19 W 7 and 31 W 1 showed minimal improvement.

Microwell tests

The tests were easy to read and the technique may even be preferable to test tubes. The following MAbs reacted with the A_{bantu} cells: 1 W 3, 5 W 8, 9 W 1, 11 W 3, 14 W 1, 14 W 2, 17 W 1, 19 W 7, 19 W 8, 19 W 10 and 31 W 1. The following failed to agglutinate the $A_{bantu}B$ cells: 7 W 3, 15 W 4, 23 W 1, 26 W 14, 28 W 1, 28 W 2 and 37 W 1.

Inhibition and inactivation

With the cells and dilutions selected, MAbs 7 W 2, 19 W 10, 23 W 1 and 34 W 1 were partially inhibited by 2 % N-acetyl-D-galactosamine. None of the MAbs were inhibited by D-galactose, alpha-L-fucose or N-acetyl-D-glucosamine. MAb 16 W 1 was not inhibited by pooled A secretor saliva and 26 W 1 and 26 W 2 were partially inhibited. MAbs 23 W 1, 34 W 1 and 37 W 1 were partially inhibited by pooled non-secretor saliva. MAbs 6 W 2, 7 W 3, 23 W 1, 26 W 13 and 37 W 1 were completely inhibited neat by DTT and 7 W 2, 11 W 3, 26 W 14, 28 W 1 and 28 W 2 partially inhibited. However, as none of them were inhibited when diluted, the finding may have been non-specific.

Absorption-elution

All the eluates had anti-A specificity and were recovered more readily from the A_{bantu} than from the A_2 cells used. They also resembled human polyclonal anti-A in their agglutination of A_1, A_2, A_1B and A_2B cells.

Other cells

None of the MAbs, except 34 W 1 which appeared to contain additional antibodies, agglutinated the A_m, A_{el}, Dantu+, O_h, T+, *Glycine soja*+, rabbit, rat, baboon or Guineapig cells. The « B » (genetically $A_{bantu}B$) cells were agglutinated by 19 W 7, 31 W 1 and 34 W 1 strongly and by 1 W 3, 5 W 8, 9 W 1, 11 W 3, 14 W 1, 17 W 1, 19 W 8, 19 W 10, 24 W 1, 26 W 1 and 26 W 2 weakly. The Tn cells were agglutinated only by 19 W 10 and 24 W 1. The sheep cells were agglutinated by 1 W 3, 9 W 1, 19 W 8, 19 W 10 and 31 W 1 and the *Vervet* monkey cells by 5 W 8. An additional antibody, anti-Le^a, was identified in MAb 5 W 8. The remaining cell samples reacted as expected with all the MAbs.

DISCUSSION

The titres and capabilities of the 30 MAbs varied widely. Many showed no variation by titre or score between the A_1 and A_2 or between the A_1B and A_2B cells. This pattern differed from human polyclonal anti-A. However, since the eluates more closely resembled the latter, the difference may be due to antibody molecule concentration. Some MAbs seemed to have anti-A_1 specificity by one technique but with another showed, by giving unexpected weak positive results, that they had broader specificities. The majority of the MAbs agglutinated the $A_{bantu}B$ cell samples strongly. Since some are known to be genetically A_2B, several examples were included, but all reacted almost equally well. It is interesting to speculate whether the MAbs agglutinated the bromelin-treated more strongly than the untreated A_{bantu} cells [1] because bromelin exposes further A antigen sites on the membranes of these cells [2], the A antigen sites already available are presented in a more favorable configuration for agglutination of [3] the B antigen affects the distribution of the A sites in genetically AB persons in such a way that they are « seen » by anti-A MAbs but not by human polyclonal anti-A. The « B » cells thought to be from a genetically $A_{bantu}B$ donor may have been phenotype B (A). Perhaps they had fewer A antigen sites than normal $A_{bantu}B$ cells ?

The avidity tests showed that the majority of the MAbs agglutinated the A_1, A_2, A_1B and A_2B cells rapidly. This is useful for determining blood types in an emergency. However, few MAbs agglutinated the A_{bantu} or $A_{bantu}B$ cells. The temperature and pH requirements of the MAbs seemed not critical but the optimum for each needs to be assessed individually. With few exceptions, the LISS tests also appeared to offer no advantage. The results with the A_{bantu} and $A_{bantu}B$ cells by automation technique, in which the cells are bromelin-treated as part of the normal procedure, were poor. This contrasted with the enhanced results seen in the two-stage bromelin titrations, especially those with the A_{bantu} cells. The inhibition by DTT was probably not a valid result. However, inhibition by A secretor saliva confirmed the anti-A specificity of the majority of the MAbs. We wondered why only 2 MAbs agglutinated the Tn cells ? These cells have N-acetyl-D-galactosamine exposed on their membranes. The MAbs which gave the best results in the whole study were 1 W 3, 9 W 1, 14 W 1, 17 W 1, 19 W 7, 19 W 8, 19 W 10 and 31 W 1.

With test tubes treated with 1 % gelatin solution, the tests were easy to read and record accurately. We believe that this technique has important advantages when using MAbs. It may even be beneficial to store the MAbs in treated glassware.

The future of anti-A MAbs seems assured. However, in Natal, where 4 % of blood donations from group A Blacks are type A_{bantu}, they need to be able to detect this phenotype.

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MAbs 1990 Workshop Studies with Monoclonal ABO, Anti-Lewis and Anti-I

PHYLLIS MOORES, ELIZABETH SMART and FRANCES GREEN; Natal Institute of Immunology and Natal Blood Transfusion Service

Monoclonal ABO antibodies

Thirteen anti-A, one anti-A_1, 18 anti-B, one anti-B(acq) and five anti-A+B were received for testing. The weak A and weak B reference cell samples were not sent. All the anti-A were found to be specific for A antigen. The titres with seven by the technique specified were in excess of 512 with A_1, A_2, A_1B and A_2B cells. However, only four agglutinated A_{bantu} and $A_{bantu}B$ cells, two reacted more strongly with A_{bantu} than $A_{bantu}B$ cells and three did the reverse. It was thought that the three latter MAbs had cross-reacted with another antigen on AB cells. Alternatively, the A antigen sites on $A_{bantu}B$ cells were sited in a more favorable steric position for binding than on A_{bantu} cells. The anti-A_1 (024) was weak; this specificity was more applicable to 022. Only two anti-B had titres in excess of 512 but seven others were in the 128-256 range. MAb 047 could not be tested as "acquired" B cells were not available. The titres with one anti-A+B were in excess of 512 with all the cells used except A_{bantu}, which it agglutinated to titre 4. Only one other anti-A+B reacted with A_{bantu} cells. In the specified avidity tests, the majority of the MAbs worked rapidly, agglutination appearing in 3-7 seconds. However, only three anti-A and one anti-A+B detected the A_{bantu} cells. By solid phase, seven anti-A, ten anti-B but none of the anti-A+B were suitable for use. Sadly, one anti-A+B worked very well except with A_{bantu} cells. In microwells, one anti-A, six anti-B and one anti-A+B appeared suitable. In inhibition tests with secretor saliva, all the anti-A and anti-B behaved as expected. The five anti-A+B were inhibited against both A_1 and B cells by A secretor saliva, but only three were similarly inhibited by B secretor saliva. The MAbs giving the best overall results were 006, 026, 037 and 050.

Monoclonal anti-Lewis

Three anti-Le^a and four anti-Le^b were received. The results were clear and unambiguous, and antigen specific cells were agglutinated strongest in our hands by one-stage enzyme techniques both in test tubes and on tiles. In saliva inhibition tests, the results with the anti-Le^b but not the anti-Le^a were difficult to understand. Among the anti-Le^b, those judged as giving the best results were 073, 074 and 075.

Monoclonal anti-I

Two MAbs were received. MAb 068 was confirmed as anti-I but 067 was an unusual form of anti-H. Both gave strong positive results with the cells used by both saline and one-stage bromelin techniques.

Second International Workshop on Monoclonal Antibodies for Human Blood Groups and Related Antigens, 1990

Report, part 1

by Phyllis Moores, Elizabeth Smart and Frances Green, Natal Institute of Immunology and Natal Blood Transfusion Service.

Ninty-six monoclonal antibody samples of various volumes were received. Their specificities included: anti-A, -A_1, -B, -B(acq), -A+B, anti-I, anti-Le^a, -Le^b, anti-D, -E, -e, -e-like, -c, anti-M, -N, anti-Lu, -In(Lu)rel, -RBC(CD44), anti-K2 and anti-Jk^b. Some samples required dilution before use. The diluent used was phosphate buffered saline pH 7,3 to which 1% sodium azide was added. Each sample, diluted and undiluted, was subdivided into two plastic test tubes. Except for anti-A_1 024 and anti-B 046 which were for storage at room temperature, one test tube was placed in the deep freeze and the other stored at 4°C for daily use.
The winter room temperature (April to July) varied from 18°C to 24°C.

ABO MAbs

Owing to an unforseen problem, the weak A and weak B reference cell samples were not received.

Titrations

The manual saline technique, as specified by the organisers, was used, with PBS pH 7,3 containing 2% BSA employed as the diluent. The red cells, which were used suspended in phosphate buffered saline pH 7,3, had the following phenotypes: A_1, A_2, B, A_1B, A_2B, A_{bantu}, $A_{bantu}B$, unspecified "weak A" and O. The results given by these and other cells with polyclonal ABO grouping reagents are available on request. Anti-B 027 was used neat as the specified 1/256 dilution failed to give positive results.

Results

Anti-A: MAbs 005, 006, 012, 013, 014, 016, 018, 019 and 020 all gave good positive results with A_1, A_2, A_1B and A_2B cells. However, only 006, 016, 018, 019 and 021 agglutinated the A_{bantu} and $A_{bantu}B$ cells. The "weak A" cells were not agglutinated. MAb 022 reacted more like anti-A_1 than anti-A.

Anti-B: All the MAbs except 025 appeared specific. The best results were obtained with 026 and 037. An unsuccessful attempt was made to acetylate some group B red cells by the method of W.J.Judd (Methods in Immunohematology, Montgomery, Miami, 1988, p167-168) to test MAb 047, as "acquired" B cells were not available.

Anti-A+B: Good results were obtained with all five MAbs and A_1, A_2, B, A_1B, A_2B and $A_{bantu}B$ cells. Only 050 and 052 agglutinated A_{bantu} cells. The "weak A" cells were not agglutinated.

Avidity Tests

The manual avidity technique specified by the organisers was used. The red cells were from the same source as those used in the ABO titrations.

Results

Anti-A: The "weak A" cells were not agglutinated. The only anti-A MAbs which agglutinated all the other A and AB cells were 006, 018 and 019. MAb 016 reacted well but did not react visibly with the $A_{bantu}B$ cells. The weakest positive result with A_2B cells was given by 009. No agglutination with any of the cells was detected with MAb 008 by this method.

Anti-B: MAbs 025, 026, 033, 036, 037, 039, 040, 041, 042 and 046 all gave good results. No reactions between 027, 028 and 047 and these cells were observed by this method.

Anti-A+B: All five MAbs reacted with A_2B cells in 5 or less seconds. The "weak A" cells were not agglutinated. The best results were given by 050. This was the only anti-A+B MAb which agglutinated both A_{bantu} and $A_{bantu}B$ cells.

Performance with additional red cells

The cells used included antibody screening, T-sensitised (with neuraminidase), *Glycine soja*+, O_h, A_{bantu}, weak B, weak AB, cord and A_x (ex SCARF). The saline method specified by the organisers was employed. Where another technique had been recommended by the MAb supplier (eg. immediate spin, saline 1 hr then spin and one-stage bromelin), this was used as well. The results given by these and other cells with polyclonal ABO grouping reagents are available.

Results

Anti-A: Only MAbs 006, 016 and 021 agglutinated the A_{bantu} cells. These MAbs also agglutinated A_x cells. All the anti-A MAbs except 009 and 022 agglutinated the weak AB cells. Among the additional techniques used, good results were seen with MAb 006 by saline 1 hr spin. This MAb also reacted the best of the four tested by slide technique, but missed one of the four A_{bantu} cells. In microwells, MAbs 016 and 021 provided the most satisfactory results. However, only MAb 006 agglutinated the $A_{bantu}B$ cells.

Anti-B: The best results were given by MAbs 026, 037, 043 and 046. The weak B cells were not agglutinated by MAbs 027, 028, 032, 033, 034, 035 and 047. Among the additional techniques used, MAb 044 worked remarkably well by immediate spin, MAb 026 by saline 1 hr spin and MAbs 026 and 036 by slide. Poor results were obtained with MAb 027 by one-stage bromelin technique. In microwells, MAbs 026, 037, 039, 043 and 046 all reacted well. As already indicated, "acquired" B cells were not available to test MAb 047.

Anti-A+B: MAbs 050 and 052 both gave good results. They agglutinated all four A_{bantu} and the A_x cells. MAb 052 also reacted well by immediate spin technique. By slide technique, 050 failed to react with the A_{bantu} cells. In microwells, 050 proved better than 052; the latter gave negative results with two of the four A_{bantu} samples.

Performance by other techniques

1. Inhibition with substances

The MAbs were diluted with PBS pH 7,3 to give an uncentrifuged 1+ result with the phenotypes selected. One volume of diluted MAb was mixed with one volume of inhibiting substance and incubated at 19°C for 30 minutes. One volume of 3-5% cell suspension was added and the test was re-incubated at 19°C for 60 minutes. The tests were then read.

Results

Anti-A: All the MAbs tested were inhibited by A secretor saliva. MAbs 012, 014, 016, 018, 022 and 024 were also inhibited by 0,1 M N-acetyl-D-galactosamine.

Anti-B: B secretor saliva inhibited all the MAbs tested except 025, 033 and 044. The sample of 0,1 M D-galactose was almost certainly inactive.

Anti-A+B: The activity of all five MAbs was inhibited by A secretor saliva, whether A_1 or B cells was used. MAbs 048, 050 and 052 were similarly inhibited by B secretor saliva, whether A_1 or B cells was used.

2. pH sensitivity

Phosphate buffered saline at pH 8,4, pH 7,2 and pH 6,0 were prepared. The saline technique specified by the organisers was employed. Only anti-A 008 and anti-B 036 were tested.

Results

The degree of agglutination seen was the same in all three pH solutions.

3. Anti-B(A) activity

As B(A) cells were not available, the anti-A MAbs were tested by saline, one-stage and two-stage bromelin technique at 20°C with light centrifugation against six random B cell samples from Black donors.

Results

No positive results were obtained. Since MAb 1 W 3 from the first monoclonal antibody workshop also gave negative results, perhaps this test should be ignored.

4. Excluding other antibodies

The saline 1 hr spin technique at 18°C was used with the nine panel cell samples and baboon and Vervet monkey red cells.

Results

No unexpected antibodies were found. Anti-A 019 and anti-A+B 052 agglutinated the baboon cells. This suggested that these MAbs contained human serum.

5. Solid phase serology

The protein concentrations of the supernatent and ascitic fluids were estimated by measuring their absorbance at 280nm. Each fluid was diluted in phosphate buffered saline (PBS) pH 7,3 to give a final protein concentration of 20$\mu\ell$/mℓ. One hundred $\mu\ell$ of each dilution was incubated in a microplate (polystyrene U well) for 2 hours at 22°C. The microplates were washed once with tris-saline-0,05% tween 20 (TST). Fifty $\mu\ell$ of a 0,2% red cell suspension was added to each well. The microplates were incubated at 22°C for 30 minutes, then washed 3 times in saline to remove non-adherent red cells. Colour was developed by the addition of 50$\mu\ell$ orthophenylene diamine substrate, and the reaction was stopped using 100$\mu\ell$ 0,5M H_2SO_4. The absorbances were read at 492nm.

Results

The MAbs other than those listed below gave very poor results.

The majority of the MAbs (25 tested) showed similar protein concentrations within the range 2-5 mg/mℓ. Two ascitic fluids, 008 and 028, had low protein concentrations of 450 μg/mℓ, but gave good performance as solid-phase reagents. MAb 033 had a very high protein concentration of 39,4 mg/mℓ. This reagent reacted with a sheep anti-human IgG conjugated to HRPO and was probably diluted in human serum. Although MAb 033 coated well to the plastic, its performance as a solid-phase reagent when tested with red cells was poor, even after affinity-purification on a Protein-A column.

Two anti-A+B MAbs, 048 and 049, reacted like anti-A_1 when bound to plastic, probably due to solid-phase interference with the antigen binding sites.

Conclusions, ABO MAbs

Only the anti-A MAb 022 and the anti-B MAb 025 had doubtful specificity. The titres of the anti-A MAbs with A_2B cells equalled or almost equalled those that they gave with A_1, A_2 and A_1B cells. In this respect, the MAbs were of much better quality than the anti-A we had tested in the first workshop. The results of the avidity tests with all the ABO MAbs in most cases were excellent, including those of the anti-A MAbs with A_2B cells. Two anti-A MAbs (014, 020), with titres of more than 512 against other cells, failed to agglutinate either the A_{bantu} or $A_{bantu}B$ cells. This must be a disadvantage, since four anti-A MAbs (006, 016, 018, 019) successfully agglutinated both phenotypes and two anti-A+B (050, 052) succeeded in agglutinating the A_{bantu} cells. The negative results given by the anti-A and anti-A+B MAbs with the weak A cells, 1477596, were not surprising, considering the weak reactions which these cells had given with polyclonal reagents. The MAbs that provided the best overall test tube and solid phase results were anti-A 006, anti-B 026 and 037 and anti-A+B 050. No comment could be made about the activity of anti-B (acq) 047 as the red cells needed to test it were not to hand.

Lewis MAbs

Anti-Le^a MAbs

Three MAbs were received. They were 069, 070 and 071. The cells used included both strong and weak Le(a+b-): for convenience, the former is shown as Le(a++b-). In addition to saline and one-stage bromelin techniques, the one-stage ficin tile technique was used as it was our method of choice with anti-Lewis reagents. In this technique, equal

volumes of MAb, 5% cell suspension and 0,25% ficin solution are placed in this order on a glass tile. The tile is then agitated and rotated in a figure of eight pattern and placed in a moist chamber for 30 minutes. The results are read by tilting the tile over a source of light.

Titrations
The titrations were made by one-stage bromelin technique at 22°C, with PBS pH 7,3 containing 2% BSA as the diluent. MAb 070 gave the best results, but the results from dilutions 1/4 to 1/32 were difficult to read as the cells stuck to the test tube glass. The cells used included A_1, A_2, B and O Le(a+b-) Le(a-b+) and Le(a-b-), O_h Le(a+b-) and Le(a-b-) and cord cells.

Results

1. by saline 1 hr spin technique at 18°C: The most satisfactory results were given by MAb 070. None of the MAbs agglutinated the cord cells.
2. by saline 1 hr spin technique at 4°C: MAbs 070 and 071 both reacted well.
3. by one-stage bromelin technique at 19-22°C: All of the MAbs gave excellent specific results. Cord cells were not agglutinated.
4. by one-stage ficin tile technique at 18°C: Excellent specific results were obtained with all three MAbs. However, stronger positive results were seen with 20% than with 5% cell suspensions.

No evidence of anti-A or anti-B specificity was detected by any of the techniques used.

Inhibition tests
MAb 069 was not inhibited. MAbs 070 and 071 were inhibited by Le^a secretor saliva. In addition, MAb 070 was inhibited by $AHLe^b$, by one of three $BHLe^b$ and HLe^b secretor saliva but not by saliva containing H substance only. Not all the salivas which contained Le^a substance inhibited MAb 071.

Conclusions, anti-Le^a MAbs
All three MAbs were specific for Le^a antigen. They also all appeared eminently suitable for use as anti-Le^a reagents by one-stage enzyme technique.

Anti-Le^b MAbs
Four MAbs were received. They were 073, 074, 075 and 076. The cells used included both strong and weak Le(a-b+): for convenience, the former is shown as Le(a-b++). In addition to saline and one-stage bromelin, the one-stage ficin tile technique (please see anti-Le^a) was used.

Titrations
The titrations were made by one-stage bromelin technique at 22°C with PBS pH 7,3 containing 2% BSA as the diluent. The cells used included A_1, A_2, B and O Le(a-b+) Le(a+b-) and Le(a-b-), O_h Le(a+b-) and Le(a-b-) and cord cells.

Results

The highest titres were those given by MAbs 075 and 076.

1. by saline 1 hr spin technique at 18°C: Very poor results were obtained.
2. by one-stage bromelin technique at 19°C: Good specific results which were easy to read were obtained with MAbs 073, 074 and 075. Cord cells were not agglutinated. MAb 076 gave weak positive results with A_1 Le(a-b+) cells.
3. by one-stage ficin tile technique at 18°C: Excellent specific results were obtained: the best were given by MAbs 073, 075 and 076. Stronger positive results were seen with 20% than with 5% cell suspensions.

No evidence of anti-A or anti-B specificity was detected by any of the techniques used.

Inhibition tests

Details of the inhibition technique are available on request. MAbs 073 and 074 were inhibited by all the salivas containing Le^b and some of those containing Le^a substances. MAb 075 was inhibited by the salivas containing H and Le^b but not by those containing AH substances. MAb 076 was inhibited by salivas containing HLe^b and $AHLe^b$ but not by those containing $BHLe^b$ substances.

Conclusions, anti-Le^b MAbs

MAbs 073, 074 and 075 gave good specific results. They appeared eminently suitable for use as anti-Le^b reagents by one-stage enzyme technique.

Anti-I MAbs

Two MAbs were received. They were 067 and 068. The cells used included adult strong and weak I+, adult I-, adult O_h Le(a+b-) and O_h Le(a-b-) and cord cells.

Results

Titrations

1. by saline 1 hr spin technique at 18-20°C: The specificity of MAb 068 appeared to be anti-I. MAb 067 reacted more like anti-H, the O_h cells giving negative results. When titrated against A_1, A_2, B and O cells, however, the O cells were the weakest, not the strongest, agglutinated.
2. by one-stage bromelin technique at 18-22°C: Similar but higher titred results were seen.

Specificity

1. by saline 1 hr spin technique at 18-22°C: MAb 068 reacted as anti-I and MAb 067 as anti-H, both in untreated and in gelatin-treated (to avoid cell sticking problems) test tubes.
2. by one-stage bromelin technique at 22°C: The results were stronger. MAb 068 again gave the results expected of anti-I and MAb 067 of anti-H.

Inhibition

Neither MAb was inhibited.

Conclusions, anti-I MAbs

MAb 068 was usable as an anti-I reagent. MAb 067 may have anti-H specificity but some of its reactions were unlike those of human anti-H.

MAbs 1990 Workshop Studies with Monoclonal Anti-M, Anti-N, Anti-Lu, Anti-RBC(CD44), Anti-In(Lu), Anti-K2 and Anti-Jk^b

PHYLLIS MOORES, ELIZABETH SMART and FRANCES GREEN; Natal Institute of Immunology and Natal Blood Transfusion Service

Monoclonal anti-M and anti-N

Five anti-M and seven anti-N MAbs were received. The specified buffer, 0,01 M sodium phosphate in 0,15 M NaCl, and the cells of specified phenotypes, together with other cells available, were used. In the titrations by specified saline technique, anti-M 130, 131 and 132 showed gene dosage and 131, 132 and 133 agglutinated the S+ stronger than the S- cells. The titres of the anti-N with Ns cells were between 8 and 256. MAbs 140 and 146 were inactive. In the specificity tests, by tube technique all the anti-M but only anti-N 139 gave specific results; the latter correctly agglutinated Mu/Dantu and M St(a+) cells. By tile technique, anti-M 130 was particularly avid and all the anti-N showed more specific results. Only anti-M 132 appeared unaffected by pH changes. The specificity of the anti-M and non-specificity of the anti-N were confirmed by immuno-blotting technique.

Monoclonal anti-Lutheran

One anti-Lu (163), one anti-RBC(CD44) (166) and two anti-In(Lu) rel (164 and 165) MAbs were received. In the titrations by saline technique, the titres with 163 were around 512 but avidity low. The titres with 166 were 32-64 and with 164 (starting dilution 1/1000) 8-16. Used neat, as it was unreactive diluted 1/500, 165 also gave titres of 8-16. By indirect antiglobulin technique (IAT) using a standard reagent, the titres with 164 and 165 were 256 and 32 respectively and avidity low. In the specificity tests by 1hr saline spin technique, as they reacted with all except AET-treated, Lu(a-b-) and In(Lu)- cells, 163 and 166 were indistinguishable from anti-Lu. Neither 164 nor 165 reacted with AET-treated, Lu(a-b-) or In(Lu)- cells. By IAT, 164 reacted with Lu(a-b-) and In(Lu)- cells and 165 with Lu(a-b-) cells. Their specificities were therefore not proved.

Monoclonal anti-K2

One MAb, 167, was received. In both the titrations and specificity tests, the best results were obtained by one-stage bromelin technique at 37°C. As expected, Kk Kp(a+b+) cells reacted weakly and A_1 KK, K_o, AET-treated and Brom-ZAPP-treated cells were not agglutinated. B KK cells were not available. The cells thought to be McLeod were agglutinated 1+ by IAT only.

Monoclonal anti-Jk^b

One MAb, 168, was received. The best results were obtained by 1hr saline spin technique at 18°C and at 37°C. Although the titres were only 2, the results were clear. The MAb was not found to agglutinate either A_1 or B Jk(a-b-) cells.

Second International Workshop on Monoclonal Antibodies for Human Blood Groups and Related Antigens, 1990

Report, part 2

by Phyllis Moores, Elizabeth Smart and Frances Green, Natal Institute of Immunology and Natal Blood Transfusion Service.

Anti-M MAbs

Five anti-M MAbs were received. They were 130, 131, 132, 133 and 134. MAb 131 was used diluted 1/100 and MAb 134 diluted 1/16, as requested. The phenotypes of the cells used were those specified by the organisers. Other cells were included as they were readily available. The cells were suspended in a solution containing 0,01 M sodium phosphate in 0,15 M NaCl, as directed by the organisers. The technique specified by the organisers was adhered to strictly throughout the study. Other techniques were included.

Results

Titrations

As directed, the MAbs were titrated in the solution containing 0,01 M sodium phosphate in 0,15 M NaCl plus 2% BSA. The best results were given by MAbs 130 and 132. The cells in the dilutions between 1/8 and 1/64 were sometimes difficult to read as they stuck to the test tube glass. M gene dosage was evident with MAbs 130, 131 and 132. MAbs 131, 132 and 133 agglutinated the S+ more strongly than the S- cells.

Specificity

In addition to the cells with the phenotypes requested, other cells with a variety of phenotypes in the MNSs system were included.

1. by saline technique at 18-20°C spun after 15 minutes: No evidence of anti-A or anti-B specificity was seen. All the MAbs agglutinated MS, MNSs and Ms cells and agglutinated M_1, M U- Dantu+, NS Dantu+ (known to have some M antigen), M St(a+), MNS Mi.II, M He+, M^g, cord and M T-sensitised (neuraminidase-treated) cells. None agglutinated Ns, N U-, N U^{var}, Ns Mi.III, N T-sensitised (neuraminidase-treated) or N *Glycine soja*+ cells. Diluted 1/100 and 1/16 respectively, MAbs 131 and 134 gave difficult to read results as the cells stuck to the test tube glass. This problem was partially overcome by repeating the tests with MAbs 131 and 134 diluted with the fluid containing 2% BSA.
2. by saline technique at 18-20°C spun immediately: MAb 130 gave excellent results. The results with MAb 134 diluted 1/16 were difficult to read as the cells stuck to the test tube glass. However, markedly improved results were obtained when MAb 134 was diluted with the fluid containing 2% BSA.
3. by tile technique read immediately and after 2-3 minutes: MAb 130 gave outstanding results! It agglutinated almost all of the M+ cells an immediate 4+. MAb 132 reacted less strongly. The positive results with MAbs 131 and 134 were weaker, but they improved when the MAbs were diluted instead with the fluid containing 2% BSA. MAb 133 worked poorly by this technique.
4. by two-stage bromelin technique: No positive results were obtained.

pH sensitivity

All five MAbs were diluted further to improve their sensitivity to pH changes. The specified saline technique was used. At pH 6,0, MAbs 130 and 131 worked poorly and MAb 133 not at all. MAbs 132 and 134 appeared unaffected. At pH 7,2, all five MAbs reacted. At pH 8,4, MAb 130 failed to react, and MAb 134 gave weaker results. MAbs 131, 132 and 133 were unaffected at this pH.

Conclusions, anti-M MAbs

The best results were given by MAb 130, but it was clearly sensitive to pH changes. MAbs 132 and 133 also reacted well, although their results were not as good as those of MAb 130 by tile technique. MAb 132 was stable at different pHs, but MAb 133 failed to agglutinate cells at pH 6,0. MAbs 131 and 134 may show improvement if diluted less or diluted with fluid containing BSA at a higher concentration than 2%.

Anti-N MAbs

Eight anti-N MAbs were received. They were 136, 137, 138, 139, 140, 141, 142 and 146. The dilution chosen for 140 was 1/6. The phenotypes of the cells were those specified by the organisers, but other cells were included because they were readily available. All the cells were suspended in the solution containing 0,01 M sodium phosphate in 0,15 M NaCl, as directed by the organisers. The technique specified by the organisers was adhered to strictly, and other techniques were included.

Results

Titrations

As directed, the MAbs were titrated in a solution containing 0,01 M sodium phosphate in 0,15 M NaCl plus 2% BSA. The best results were given by MAb 139. MAbs 136, 137, 138, 141 and 142 showed evidence of non-specificity. MAbs 140 and 146 were inactive.

Specificity tests

In addition to the cells with the phenotypes requested, cells with a variety of phenotypes in the MNSs system were included.

1. by saline technique at 18-19°C spun after 15 minutes: All the MAbs agglutinated MNS and Ns cells and agglutinated N U-, N U^{var}, MN and N *Glycine soja*+, MNS Mi.II, Ns Mi.III, MU-/Dantu+, NS/Dantu+, MNS He+, M_1, M^g, M and N T-sensitised, M St(a+) and cord MNs cells. However, only MAb 139 gave specific results. It also showed no evidence of anti-A or anti-B specificity. Mixed fields were obtained with MAb 137 and A_1 MS and B Ms cells. MAb 140, used both neat and diluted 1/6, reacted poorly. MAb 146 was inactive. MAb 139 gave negative results with M T-sensitised, M_1 and M^g cells. The positive results obtained with this MAb and MU-/Dantu+ and M St(a+) cells were particularly important. The agglutination with MAbs 136, 138, 139, 141 and 142 was firm but with MAbs 137 and 140 (neat) loose.
2. by tile technique read immediately and after 2-3 minutes: Using approximate 10% cell suspensions, the MAbs showed much better evidence of anti-N specificity. However, MAbs 140 (neat) and 146 were still inactive. The strongest positive results were given by MAb 142 at 2-3 minutes. The avidity improved slightly with 20% cell suspensions.

3. by two-stage bromelin technique: Only MAb 140 gave positive results; however, they were not specific for N.

pH sensitivity
All eight MAbs were diluted further to improve their sensitivity to pH changes. The specified saline technique was used. MAbs 136 and 137 only gave positive results at pH 8,4. MAbs 138 and 139 worked best at pH 7,2 and MAb 142 best at pH 6,0.

Conclusions, anti-N MAbs
By saline tube technique, only MAb 139 had anti-N specificity; nevertheless, it was clearly sensitive to pH changes. None of the other MAbs were satisfactory as anti-N reagents.

Immuno-blotting technique

Solubilised red cell membranes and neuraminidase-treated red cell membranes were prepared from the blood of genetically homozygous M and N individuals. The glycoproteins were separated on SDS-PAGE and electrotransferred onto cellulose nitrate membranes. Immuno-blotting was performed. The protein bands were visualised using Biotin- labelled sheep anti-mouse IgM and rabbit anti-mouse IgG coupled with Strep-Avidin conjugated to HRPO.

Results

Anti-M MAbs: All five MAbs reacted with bands corresponding to α_2, $\alpha\delta$ and α on M+N- but not on M-N+ membranes. MAbs 130, 131, 132 and 134 showed reduced activity on neuraminidase-treated M+N- membranes. MAb 133 showed no reduction in activity.

Anti-N MAbs: MAbs 140, 141, 142 and 146 showed weak or no bands at all by this method. MAbs 137, 138 and 139 reacted with bands corresponding to α_2, $\alpha\delta$, δ_2, α and δ on M-N+ membranes and to $\alpha\delta$, δ_2 and δ on M+N- membranes. MAb 136, in addition to these bands, reacted weakly with α_2 and α on M+N- membranes. The results with the neuraminidase-treated membranes and MAbs 137, 138 and 139 were weak, although 137 and 138 showed slightly enhanced reaction with the δ_2 band on M+N- membranes. MAb 136 did not show reduced activity with neuraminidase-treated membranes; however, it again showed increased staining intensity with the δ_2 band on M+N- membranes.

Anti-Lu and anti-RBC(CD44) MAbs

One anti-Lu MAb, 163, and one anti-RBC(CD44) MAb, 166, were received. They were both tested with the same cells (phenotypes in the Lutheran system). All the panel cells were from males and had been typed for Xg^a.

Results

Titrations
The saline 1 hr spin technique was employed, using PBS pH 7,3 plus 2% BSA as the

diluent and PBS pH 7,3 to suspend the cells. The titres with MAb 163 were 512 with almost all the panel cells. However, the avidity of this MAb was only 1+ from the 1/16 to 1/256 dilution. MAb 166 gave moderate titres of 32-64 with all the cells used.

Specificity
MAbs 163 and 166 agglutinated all except the Lu(a-b-), In(Lu)- and AET-treated cells by saline 1 hr spin technique at 20°C. No changes in avidity were seen with cells of different Xg^a phenotypes.

Conclusions, anti-Lu and anti-RBC(CD44) MAbs
The specificity of both MAb 163 and 166 appeared to be anti-Lu. The cells with special phenotypes needed to distinguish anti-RBC(CD44) from anti-Lu were unfortunately not available.

Anti-In(Lu)rel MAbs

Two MAbs, 164 and 165, were received. MAb 164 was diluted 1/1000 and 165 1/500 with PBS pH 7,3 as directed. However, when MAb 165 was found to be unreactive, it was employed neat. The cells used had various phenotypes in the Lutheran and P systems. Other cells were included.

Results

Titrations
The titrations were made using PBS pH 7,3 plus 2% BSA as the diluent.

1. by saline 1 hr spin technique: The results with MAb 164 were poor, and with MAb 165 moderate (titre 8-16).
2. by indirect antiglobulin technique: The standard antiglobulin reagent containing anti-IgG+complement was used. Moderate titres were obtained, but the avidity was only 1+ to 2+.

Specificity

1. by saline 1 hr spin technique at 18-20°C: Neither MAb 164 nor MAb 165 agglutinated Lu(a-b-), In(Lu)- or AET-treated cells. In addition, MAb 164 failed to react with T-sensitised cells.
2. by indirect antiglobulin technique: MAb 164 agglutinated Lu(a-b-), reacted weakly with In(Lu)- and failed to react with AET-treated cells. The positive results were difficult to read as the cells stuck to the test tube glass. This was overcome by diluting the MAb 1/1000 in PBS pH 7,3 plus 2% BSA. MAb 165 agglutinated Lu(a-b-) but not In(Lu)- or AET-treated cells.

Conclusions, anti-In(Lu)rel MAbs
MAb 164 was obviously a powerful reagent. However, its specificity and that of 165 as anti-In(Lu) was not established beyond doubt.

Anti-K2 MAb

One MAb, 167, was received. The cells used included Kk Kp(a+b+), Kk Kp(a-b+), KK, K_o, AET-treated and a sample with depressed Kell antigens which was thought to be phenotype McLeod.

Results

Titrations
The best results were obtained by one-stage bromelin technique at 37°C. As expected, the Kk Kp(a+b+) cells were agglutinated weakly.

Specificity
No positive results were obtained by saline technique. By one-stage bromelin technique at 37°C, good specific results and no evidence of anti-A specificity were seen. Group B K_o cells were not available. Baboon and Vervet monkey red cells were not agglutinated, showing that MAb 167 probably did not contain human serum. Tested by indirect antiglobulin technique and using the standard antiglobulin reagent, the MAb gave weaker results. Improved results were obtained when rabbit mouse antiglobulin reagent (Lot IC-17) diluted 1/100 with saline was employed.

Conclusions, anti-K2 MAb
MAb 167 worked very well as an anti-K2 reagent by one-stage bromelin technique at 37°C.

Anti-Jk^b MAb

One MAb, 168, was received. The cells used included Jk(a-b-) as well as those with common Kidd phenotypes.

Results

Titrations
The diluent used was PBS pH 7,3 plus 2% BSA. By saline 1 hr spin technique at 18°C, MAb 168 gave titres of 2 with Jk(b+) cells. At 37°C, the titres were slightly higher. When complement was added to the test, the titres remained the same but the avidity decreased.

Specificity
Specific results were obtained by saline 1 hr spin technique at both 18°C and 37°C. No evidence of anti-A or anti-B specificity was seen. Some evidence of Jk^b gene dosage was also observed. By one-stage bromelin technique, the results were also specific, but more varied agglutination was seen by indirect antiglobulin technique using the standard antiglobulin reagent. Using rabbit anti-mouse antiglobulin reagent IC-17 diluted 1/100 with saline, no improvement was noted, and the agglutination was less avid.

Conclusions, anti-Jk^b MAb
Although low titre, MAb 168 appeared to be a very good reagent. The best technique was saline at room temperature. The MAb was not inactivated by bromelin.

MAbs 1990 Workshop Studies with Monoclonal Anti-Rh

PHYLLIS MOORES, ELIZABETH SMART and FRANCES GREEN; Natal Institute of Immunology and Natal Blood Transfusion Service

Monoclonal anti-D

Four IgM and 22 IgG anti-D MAbs and one reference anti-D sample were received. The three D^u and one Category VI reference cell samples were not sent. Dr Tippett graded one of our cells as D^u Category VII (G+V-VS-Tar+) and the other as on the border line of average and high grade D^u (G+V-VS-Tar-). In the specified saline (ss) titrations, three IgM MAbs had titres of 32-64 with R_1r cells and the fourth a titre of 256-512. In the specified indirect antiglobulin (IAT) titrations, using an anti-IgG + complement reagent, ten IgG MAbs had titres of over 512 with R_1r cells, and only two were low. Ten IgG MAbs were titrated by one-stage bromelin technique, and all showed substantially reduced titres. Little change was seen with five IgG MAbs titrated by LISS-IAT. The MAbs were all specific for D antigen. Only 093 among the IgM detected (ss) both graded D^us but missed others. All except 084, 089 and 105 of the IgG detected (IAT) both graded D^us, but only 083, 101 and 107 detected them. All the IgM and IgG anti-D reacted with the three R^{od} and two R_o^{dg} cells (084 missed one R_o^{dg}). Therefore none had anti-Rh^D specificity. By one-stage bromelin technique, nine IgG MAbs tested gave weak positive results, particularly with the D^us, and one was non-specific. MAb 095 gave negative results. With cells suspended in LISS, only the IgM reacted. By solid phase technique, except 084 and 093, every MAb was suitable for use.

Monoclonal anti-c

One IgG MAb, 128, was received. The titre with R_1r cells by IAT was 128 and by one-stage bromelin technique 8. Type r'^s cells were detected by both techniques. The anti-c specificity was confirmed; pH sensitivity was not detected.

Monoclonal anti-E

One IgM MAb, 125, was received. The titre with R_2r cells by 1hr spin saline technique at 37°C was 128. The specificity was confirmed by both saline and one-stage bromelin techniques with, among others, r"r", R_ZR_1, R_ZR_2 and R_2R_2 cells. At pH 6,0 only negative results were obtained.

Monoclonal anti-e and anti-e-like

Two anti-e (IgM 127 and IgG 126) and one anti-e-like (IgM 129) MAbs were received. Their titres with r"r cells by 1hr saline spin technique at 37°C were 1, 1 and 2 resp. but 256, 2 and 2 resp. with r'^s cells. By IAT, 126 reacted to titre 1 with r"r cells, and by one-stage bromelin technique to titre >512. Extensive tests eliminated as possible alternatives anti-hr^S, -hr^B, -Rh18 and -Rh34. By 1hr saline spin technique with r"r cells at 37°C, 126 was negative at pH 8,4. At 20°C, 127 and 129 were negative at pH 6,0.

Second International Workshop on Monoclonal Antibodies for Human Blood Groups and Related Antigens, 1990

Report, part 3

by Phyllis Moores, Elizabeth Smart and Frances Green, Natal Institute of Immunology and Natal Blood Transfusion Service.

MAbs in the Rh system

Owing to an unforseen problem, the three D^u and one Category VI reference cell samples were not received.
Dr Tippett graded our cells 1522447 as D^u category VII (G+ V- VS- Tar+) and our cells 1501802 as being on the border line of average and high grade D^u ($G+^W$ V- VS- Tar-).

Anti-D MAbs

Titrations

The manual saline technique for the IgM anti-D and manual indirect antiglobulin technique for the IgG anti-D MAbs were employed as directed. Phosphate buffered saline pH 7,3 containing 2% BSA was used as directed as the diluent. The antiglobulin reagent was from the routine NBTS laboratory stock and contained anti-IgG+complement components. It had been prepared and standardised in accordance with the requirements of the American Food and Drug Administration's Bureau of Biologics. The cells used had the phenotypes specified by the organisers. Other techniques employed were those recommended by the MAb suppliers. The other cells used were those we considered most likely to provide meaningful results.

Results

1. by specified saline technique: Of the four MAbs tested, 085, 093 and 110 gave titres of 32-64 with R_1r cells. The titre with the reference sample, MAD-2, was 16-34. The highest titre, 256-512, was obtained with MAb 111.
2. by specified indirect antiglobulin technique: The titres given by all 22 MAbs tested ranged from 4 to more than 512; ten achieved the latter value. MAb 090 was hard to assess as the cells stuck to the test tube glass. Consequently, it was presumed to have been diluted before receipt. The best overall results were given by MAb 107. However, the avidity of MAb 091 was a remarkable 3+ to titre 128 and that of MAb 112 3+ to titre 32.
3. by other techniques:
   (a) one-stage bromelin: AB serum was used as the titration diluent as 2% BSA provided the system with insufficient protein to promote agglutination at dilutions higher than 1/8. Ten anti-D MAbs and the MAD-2 control were titrated. MAb 091 and MAD-2 gave titres of 256 and MAb 107 a titre of 512. MAb 095 provided a disappointingly weak result.
   (b) LISS + indirect antiglobulin: Five MAbs were titrated. The titres with four and MAD-2 were 256-512.

Specificity tests

The techniques specified by the organisers were used. The cells, suspended in PBS pH 7,3, included the nine panel, A_1 r and B r, a fresh R_1R_1 and a local low and a local high grade D^u. Other techniques recommended by the MAb suppliers were also included. These were the slide at 37°C, one-and two stage bromelin at room temperature and LISS-indirect antiglobulin techniques.

Results

1. by specified saline technique: The results with all four MAbs tested showed that they were specific for D. MAb 083 was also tested as it was said to give "direct agglutination", but the results were negative. The best performances were given by MAbs 093 and 110, which were the only MAbs to agglutinate the local low grade D^u cells.
2. by specified indirect antiglobulin technique: The results showed that all 22 MAbs tested were specific for D. However, 090 and 104 proved a problem to read as the cells stuck to the test tube glass. MAbs 090, 100 and 112 missed the local D^u, and MAbs 084, 089 and 105 missed both local D^u cells.
3. by slide technique: Four MAbs were tested but only 085 reacted. It gave negative results with both D^u cells.
4. by one-stage bromelin technique: Among the ten MAbs tested, eight showed D specificity and one was inactive. The ninth MAb, 090, also agglutinated A_1 r and B r cells. All the MAbs except MAD-2 failed to agglutinate the local low grade D^u cells. MAb 095 gave negative results with all the cells used.
5. by two-stage bromelin technique: MAb 089, alone among the ten tested, agglutinated O r cells. The remainder were specific for D. However, none reacted with the local low grade D^u cells.
6. by LISS + indirect antiglobulin technique: Five MAbs were tested and all gave specific results with all the cells including the two local D^us.

Performance with additional red cells

The cells used to test the IgM MAbs included R_o type Rh:-19 (hr^S-), R_o type Rh:-34 (hr^B-), Dantu+ (D↑), En^a/En (D↑), R_o^{dg}, three R_o^d, R_o type D^u He+, r''^Gr, four R_o type low grade D^u (one particularly low), one R_1r type D^u, one R_2r type D^u, and baboon and Vervet monkey cells. Those used to test the IgG MAbs included all of the above, three further R_o type D^u among which was 1501802 graded by Dr Tippett, two further R_1r type D^u among which was 1522447 graded by Dr Tippett, and a further R_o^{dg}. The results given with polyclonal anti-Rh grouping reagents by some of these cells are available.

Results

1. by specified saline technique: The four MAbs tested gave specific results but failed to agglutinate many of the D^u samples. However, they all reacted with all three R_o^d and the two R_o^{dg} cells. The best results were given by 093.
2. by specified indirect antiglobulin technique: Among the 22 MAbs tested, 083, 101 and 107 provided excellent and specific results and even agglutinated the particularly weak D^u cells. All 22 MAbs also gave positive results with all three R_o^d and the two R_o^{dg} cells. The Vervet monkey cells were agglutinated weakly by 17 MAbs; this may show that they contained material of human origin.
3. by one-stage bromelin technique: Ten MAbs were tested. Many failed to

agglutinate the D^u samples. MAb 098 gave the best results and 095 was inactive.

4. by LISS only technique, at 37°C: Twenty-six MAbs were tested. The best results were obtained with 085, 093, 110 and 111. MAb 111, by agglutinating both baboon and Vervet monkey cells, showed that it probably contained human serum.

Solid phase serology (for method, please see ABO in part 1)

Results

With the exception of 084 and 093, all anti-D MAbs were suitable as solid-phase reagents. The IgG MAbs were slightly better than the IgM MAbs, insofar as they detected the low grade Dus.

Conclusions, anti-D MAbs

It was a pleasure to test these MAbs! Except for the problems with reading when the cells stuck to the test tube glass, clear positive and negative results were obtained. All the MAbs including 109 were tested with R_o^d and R_o^{dg} cells. The results showed that none had anti-Rh^D or anti-G specificity. MAb 095 was not suitable for use by bromelin technique. The anti-D MAbs giving the best overall results in test tubes were 093 (IgM) and 091 (IgG), closely followed by MAb 107. By solid phase technique, with the exception of 084 and 093, all the MAbs were suitable for use as anti-D grouping reagents.

MAbs with other Rh specificities

The methods and fluids used and the phenotypes of the red cells were all those specified by the organisers. Where left to our discretion, the standard techniques in current use in the laboratory were employed. When a particular technique was recommended by the MAb supplier, this was included. The antiglobulin reagent was from the routine NBTS laboratory stock and contained anti-IgG and anti-complement components. It had been prepared and standardised in accordance with the requirements of the American Food and Drug Administration's Bureau of Biologics. Other cells used were those we considered most likely to provide meaningful results.

Anti-c MAb

One MAb, 128, was received.

Results

Titration

The titrations were made by one-stage bromelin and indirect antiglobulin technique. The titre by bromelin technique with R_1r cells was 8 and avidity poor. By the antiglobulin technique, the titre was 128 and avidity excellent. With r'r and homozygous r'^s cells, the titres were 16 and 64 respectively by the bromelin and more than 512 and 512, respectively, by the antiglobulin technique. The antiglobulin technique was therefore the method of choice.

Specificity
MAb 128 appeared specific for anti-c by both saline, one-stage bromelin and indirect antiglobulin techniques. The avidity by saline and bromelin techniques was weaker than by antiglobulin technique. By both saline and antiglobulin techniques, homozygous r's cells were agglutinated strongly. The MAb showed no evidence of anti-A or anti-B specificity.

pH sensitivity
Tested by indirect antiglobulin technique, no change in avidity was noted when MAb 128 was diluted 1/64 in and the cells suspended in PBS pH 8,4, pH 7,2 or pH 6,0.

Conclusions, anti-c MAb
MAb 128 appeared a splendid anti-c reagent. It is recommended for use by indirect antiglobulin technique.

Anti-E MAb

One MAb, 125, was received.

Results

Titration
The techniques used were saline at 37°C for 60 minutes then spin and saline at 21°C for 5 minutes then spin. By the former technique, MAb 125 reacted to titre 128 with R_2r and r"r and more than 512 with R_2R_2 cells. By the latter technique, it reacted to titre 16 and 64 respectively with these cells. R_ZR_1 cells were agglutinated more strongly at 21°C (titre 4) than at 37°C (titre 1). The reason for this was not known.

Specificity
Strong specific results were obtained by saline technique both at 37°C and at 21°C. The results were also very good by one-stage bromelin technique at 37°C and 20°C. Type R_ZR_1, R_ZR_2 and r"r" cells were included in these studies. The MAb showed no evidence of anti-A or anti-B specificity.

pH sensitivity
Diluted 1/20 with PBS pH 6,0 and tested by saline technique at 37°C, MAb 125 failed to react with r"r cells suspended in this buffer. It reacted strongly when PBS pH 7,2 or pH 8,4 was substituted.

Conclusions, anti-E MAb
MAb 125 was judged to be an excellent anti-E reagent when used by saline technique. Tests at room temperature appeared preferable, even though the titre was lower than at 37°C.

Anti-e and anti-e-like MAbs

Two anti-e (126, 127) and one anti-e-like (129) MAbs were received. Our studies included tests for anti-hr^S (-Rh19), anti-Rh18, anti-Rh31 (-hr^B) and anti-Rh34 specificity. An explanation may be needed here for clarity. Anti-hr^S (-Rh19) and anti-hr^B (-Rh31) are reagents "manufactured" by man. This is done by absorbing sera containing anti-Rh18 and

anti-Rh34 with R_2R_2 cells (Shapiro: J. forens. Med. 7, 96, 1960; Shapiro, Le Roux and Brink: Haematologia 6, 121-128, 1972). Neither anti-hr^S (-Rh19) nor anti-hr^B (-Rh31) are therefore likely to occur naturally. A paper explaining this in more detail is in preparation (Moores and Smart, 1991). Red cells labelled hr^S- and hr^B- in fact are Rh:-18 and Rh:-34, respectively, but R_2R_2 and other E/E cells have weakly expressed Rh18 and Rh34 antigens. Phenotype Rh:-18 and Rh:-34 red cells occur relatively frequently in the Natal Blacks. Red cells other than those with "deleted" and Rh_{null} phenotypes therefore give negative results with anti-Rh18 and anti-Rh34.

Results

Titrations

1. by saline 1 hr spin technique at 37°C: The titres given by MAbs 126 and 129 with the cells used were low (1-2). MAb 127, although giving equally weak results with r"r and r cells, reacted to titre 4-8 with R_o type Rh:-19 (hr^S-) and 256 with r'^s type Rh:-34 (hr^B-) cells.
2. by saline 1hr spin technique at 18°C: The results with 127 were similar to those obtained at 37°C. The strongest positive findings were with the C+ cells.
3. by indirect antiglobulin technique: The titre given by MAb 126 was low (1-2).
4. by one-stage bromelin technique: MAb 126 reacted to much higher titres by this method than by saline or antiglobulin techniques. The titres were more than 512 with r, r"r and R_1R_1 and 16 with R_2R_2 cells. MAb 127 reacted weakly; the titres were 4-8 and 16 with these cells respectively. MAb 129 reacted to titre 64 with the first three but only to titre 1 with R_2R_2 cells.

Specificity

Three R_o type Rh:-19 (hr^S-), three R_o type Rh:-34 (hr^B-), three r'^s type Rh:-34 (hr^B-) and one each of r', R_ZR_2 and A_1, B and O R_2R_2 cells were used.

1. by saline 1 hr spin technique at 18-20°C: MAb 126 gave weak positive results. The C+ appeared to be agglutinated stronger than the C- cells. MAbs 127 and 129 agglutinated all except the E/E cells. None of the MAbs showed evidence of anti-A or anti-B reactivity.
2. by saline 1 hr spin technique at 37°C: MAb 126 reacted more strongly by this method and showed anti-e specificity. Except with the hr^S- and Rh:-34 cells, MAb 127 also looked more like anti-e. The results with MAb 129 were not consistent for anti-e as it failed to react with some but not all the hr^S- and Rh:-34 cells.
3. by saline tests at 4°C: MAb 126 was unreactive.
4. by slide technique at 37°C: the results given by MAb 126 agreed with an anti-e specificity.

pH sensitivity

By saline 1 hr spin technique at 37°C, MAb 126 diluted 1/2 with PBS pH 8,4 or pH 7,2 was unreactive with r"r cells suspended in these buffers. However, it reacted weakly with these cells when diluted with PBS pH 6,0. MAbs 127 and 129 diluted 1/2 with PBS pH 6,0 failed to react with these cells, but did react with them when diluted similarly with PBS pH 8,4 or pH 7,2.

Conclusions, anti-e and anti-e-like MAbs

Except by one-stage bromelin technique, where non-specific results were obtained, MAb 126 appeared to have anti-e specificity. A decision on the best method for its use was

difficult. MAb 127 looked more like anti-Ce than anti-e. While 127 reacted strongly with C+ cells, its specificity was clearly not anti-Rh34. MAb 129 was rather weak but did resemble anti-e in its reactivity.

General conclusions about all the MAbs in Reports 1, 2 and 3

The ABO, anti-Le^a, -Le^b, -I, -D, -c, -E, -M, -Lu, -K2 and -Jk^b MAbs were all a pleasure and great privilege to use. They are sure to become most useful serological tools. The anti-e and anti-N MAbs were disappointing and the anti-In(Lu)rel MAbs relatively so. We did not feel sufficiently competant to comment on the activity of the anti-RBC(CD44) MAb.

References

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CHAPTER XI

TECHNIQUES

XI.1 PAPERS

19S Rhesus antibodies in Rh sensitized Bantu and European women in Natal
Paper 13 by Moores and Grobbelaar

A screening test for antibodies using 2-mercaptoethanol
Paper 15 by Moores and Grobbelaar

Haemolytic disease of the newborn due to ABO incompatibility - a new aid to diagnosis
Paper 18 by Moores, Grobbelaar and Ward

Some problems of Rh grouping by the Chown technique
Paper 17 by Dobie and Moores

XI.1 INTRODUCTION

In 1944, Diamond reported a prozone effect with a sample of Rh D antibodies. Later that same year and working independently, both Race and Wiener discovered that the prozone was due to "hidden" anti-D. Race called the antibody incomplete and Wiener called it blocking; in some way the anti-D was believed to be defective. After 1945, incomplete antibody detection was greatly improved by the antiglobulin test of Coombs, Mourant and Race [1945, 1946]. They have since been subdivided into two types, IgM and IgG. The IgM type was formerly known as 19S and the IgG type as 7S; these figures were their respective sedimentation rates in a G-200 sephadex column [Mollison, 1983, p215].

Type 7S (IgG), but not type 19S (IgM), Rh antibodies are known to pass through the placenta membranes into the fetus. Once there, they may cause haemolytic disease of the newborn. The 7S form of anti-A and anti-B is difficult to distinguish serologically from the 19S form, however, and both agglutinate red cells with the corresponding antigens. Several different techniques have been designed to help make the distinction [Issitt, 1985, p588-589], but so far none has proved sufficiently reliable.

In the Chown saline capillary technique for D-typing red cells [Chown, 1944], one volume of an IgM anti-D reagent is taken up in a 90mm x 0,4mm glass capillary tube of known bore. The anti-D is immediately followed, with no air bubble between them, by one volume of a 20-30% saline suspension of washed red cells from the blood sample to be tested. The capillary is inverted and placed at a 45° angle, with its end embedded in a narrow tray of plasticine against, but not touching, the illuminated glass panel of a special Chown box at 37°C. The red cells fall by gravity through the anti-D, and readings are made from 5 minutes to a maximum of 60 minutes. A positive result is indicated by the red cells having a granular appearance, and a negative result by no evidence of red cell granulation.

XI.3 COMMENTARY

XI.3.1 Attainments in Durban, 1961 to 1991

XI.3.1.1 Persistence of 19S anti-D

It is generally accepted that 19S antibodies appear first in the serum and 7S antibodies later; the 19S form then gradually disappears. In paper 13, the authors reported that substantially more Black than White women with anti-D titres of 32 and above had 19S antibodies. They put forward several possible reasons for this: (1) 19S anti-D might persist longer in Black than White women; (2) Black women might make type 7S anti-D earlier; (3) the immune response of Black might differ from that of White women, for D antigen in Blacks was known to be stronger than in Whites and, (4) since Black generally had more stillbirths than White women, 19S antibodies might reappear in any case.

XI.3.1.2 Uses of 2-mercaptoethanol

XI.3.1.2.1 In screen testing sera for antibodies

The Natal Blood Transfusion Service employed a routine screening procedure to detect blood group antibodies in

antenatal and donor sera. In this procedure, the sera were first tested by one-stage bromelin enzyme technique against two group O red cell samples. As far as possible, the samples included all the more usual blood group antigens in the homozygous state, even if each sample had to contain blood from two people. When a serum gave a positive result, and/or the patient or donor gave a history of miscarriages, stillbirths, neonatal deaths and/or transfusion reactions, it was also tested against the screening cells by the indirect antiglobulin technique. Any antibodies detected were then identified by testing the serum against a panel of selected red cells.

The antibodies in many antenatal and donor sera were found to give positive results by the bromelin but negative results by the indirect antiglobulin technique. Moreover, no evidence that the infants of the mothers with these antibodies had suffered from haemolytic disease of the newborn was detected. The bromelin technique therefore detected many clinically insignificant antibodies. In order to avoid the considerable labour and expense of testing large numbers sera by the antiglobulin technique, the authors designed a rapid, simple technique which would eliminate the clinically insignificant antibodies [paper 15]. The technique employed 2-mercapto-ethanol, a substance which was known to dissociate type 19S (IgM) but not type 7S (IgG) immunoglobulins. Claims had been made that the substance had to be removed from the serum by dialysis or vacuum extraction before the serum could be used in serological tests. Watson [1964], however, found that the 2-mercaptoethanol did not interfere with the antiglobulin reaction. In the authors' technique, one volume of a 0,1 Molar solution of 2-mercaptoethanol was mixed with one volume of the serum to be tested. After gentle agitation, the mixture was incubated at 37°C for one hour. Without removing the 2-mercaptoethanol, one volume of a 2% saline suspension of bromelin-treated screening cells (equal volumes of washed, packed red cells and 0,5% bromelin for 10 minutes at 37°C) was then added and the tests were left on the bench at room temperature. One hour later, the results were read, without prior centrifugation, macroscopically over a source of light.

A sample of 20 273 sera was investigated both by this the normal routine technique. Excellent results were obtained. The number of bromelin screen tests which were positive decreased from 15,9% to 1,4%, and all the clinically significant antibodies identified by the normal routine technique were detected.

XI.3.1.2.2 In ABO haemolytic disease of the newborn

After exposing the sera of 53 pregnant women to anion exchange chromatography, Kochwa, Rosenfield, Tallal and Wasserman [1961] found that the ABO antibodies of five resisted inhibition by commercial ABH blood group substances. Four of the five women subsequently gave birth to group O infants and the fifth to a group B infant who had required exchange transfusion for haemolytic disease of the newborn. The technique of Kochwa et al. [1961] appeared to be ideal for identifying clinically significant ABO antibodies but, as the results were not available for two days, it was not suitable for rapid diagnosis. In paper 18, the authors described another technique in which the serum was first mixed with an equal volume of a 0,1 Molar solution of 2-mercaptoethanol. After agitation, the mixture was incubated at 37°C for one hour. Without removing the 2-mercapto-ethanol, the mixture was then diluted to 1 in 16 with saline. To one volume of the diluted mixture, one volume of diluted pooled A secretor saliva, and to another volume, one volume of diluted pooled B secretor saliva (maximum inhibition titre with the undiluted saliva: 1 in 32 000) was then added. Thirty minutes later, one volume of bromelin-treated group A red cells was added to the test tube containing the A secretor saliva and one volume of bromelin-treated group B red cells to the test tube containing the B secretor saliva. The results were read after a further one hour without prior centrifugation. The new technique required a maximum delay of two and a half hours before the results were known. The findings in the subsequent studies confirmed those of Kochwa et al.[1961] that the mothers of infants with haemolytic disease of the newborn due to ABO antibodies were more likely than the mothers of unaffected infants to have 7S

(IgG) anti-A and/or anti-B which were not inhibited by ABH substances.

XI.3.1.3 Typing red cells for D^u

Paper 17 described the authors' study made to determine why an unusually large number of blood samples from Blacks, especially those received unrefrigerated from the more remote areas of Natal, typed as D^u. For some years, the laboratory had preliminarily Rh D-typed all its blood samples by the Chown capillary technique. The definition of D^u used was that of Wiener and Wexler [1958, p85]: red cells which gave negative results with potent saline-reacting (IgM) anti-D and gave positive results with some but not all enzyme- and antiglobulin-reacting (IgG) anti-D reagents were type D^u. For three days a week during a four week period, all the blood samples that had been routinely typed by the laboratory staff as D^u and some controls were carefully retested with selected anti-D reagents by different techniques. The findings showed that the majority of the D^u were at the lower end of the normal range for D+ samples. The authors described them in paper 17 as low grade D+, but this term is now used to describe the weaker examples of D^u). Although the so-called D^u samples gave negative results by Chown technique, in test tubes they gave weak positive results with potent saline-reacting anti-D reagents. Furthermore, as the samples aged, their results weakened. The anti-D reagents had not identified the products of different genes but had differing D-typing capabilities.

Paper 13

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19S RHESUS ANTIBODIES IN RH SENSITIZED BANTU AND EUROPEAN WOMEN IN NATAL

By P. MOORES and B. G. GROBBELAAR*†

The human body contains many kinds of proteins which, together with fats, carbohydrates, mineral salts and water, go to make up the substance of the various tissues. When a foreign protein (or antigen) is introduced, the body protects itself by making an antibody with the specific task of destroying and thus removing that protein. In blood transfusion and antenatal investigations the most important antigens are those on the surface of red blood cells which may either be deliberately introduced by transfusion or accidentally acquired through the leaking of foetal cells into the maternal circulation at birth or before it.

Fortunately, red cells are not usually recognised as foreign proteins by the body or blood transfusion would become an impossibility, and only one in approximately 200 mothers has this sort of trouble with her pregnancy.

When an antibody is detected it must first be identified and it is then usually titrated using the method of increasing doubling dilutions. This is a technique used to estimate its strength and the strength is then given a numerical value called the titre.

95% of all antibodies produced by human beings in response to human red cells are Rhesus antibodies of the type known as anti-D or Rh_0. They are found in two forms — 19S or complete and 7S or incomplete. The 19S variety is capable of agglutinating red cells visibly in a saline medium, but 7S antibodies form only a sort of "coat" round each cell and no agglutination can be seen except under special conditions, such as a high protein medium.

It is necessary to distinguish between 7S and 19S antibodies because 7S are implicated far more often in haemolytic disease of the newborn. In this condition a maternal antibody attacks the foetal cells in utero, leading to jaundice and severe anaemia. A substance known as 2-mercapto-ethanol, a derivative of alcohol, has the property of making this necessary distinction by destroying the disulphide bonds of the 19S antibodies, thereby rendering them inactive. In earlier years 2-mercaptoethanol had been used only for fairly large volumes of serum but had the unfortunate disadvantage that it required to be removed again by a lengthy process of dialysis before any further tests could be done, besides, it has an awful smell. Consequently the technique had been discarded as being too unwieldy for rapid laboratory tests.

*The Natal Blood Transfusion Service, Prince Street, Durban.
†Presented at the First Scientific Congress of the Society od Medical Laboratory Technologists of S.A., July, 1968.

TABLE 1

Rhesus Antibodies in Bantu and European Women

I. Bantu

	number.	%
No I9S:	50	79·3
I9S Present:	I3	20·7
Total:	63	100

II European

	number	%
No I9S:	54	80·6
I9S Present:	I3	19·4
Total:	67	100

A modified technique[1] was introduced by the authors at the Blood Transfusion Congress at Port Elizabeth in 1966, whereby O.1 molar 2-mercaptoethanol in saline was introduced directly into the test tube, volume for volume with the patient's serum, and did not need to be removed subsequently. After a period allowed for inhibition to take place the cell suspension was added to the tube and 7S antibodies were detected by agglutination, whereas 19S antibodies remained completely inactive. Control tests were done without 2-mercaptoethanol, thus we can tell whether the antibody is pure 19S and harmless, or contains a dangerous 7S component.

It has long been believed that in resonse to an initial episode of stimulation the first kind of antibody produced is 19S, though Murray[2] has recently had certain reservations about this. 7S antibodies are thougl to appear in the serum later, probably as the result of further stimulations, and at this later stage 19S antibodies decrease gradually to insignificance or disappear altogether.

Our studies in Natal had suggested that Bantu women with 7S antibodies had an unusually high incidence of 19S antibodies as well, and we therefore decided to compare a series of Bantu women with one of Europeans.

63 Bantu and 67 European Rhesus sensitized mothers were selected by alphabetical order only, and any that had undergone diagnostic amniocentesis were discarded as this process might have caused some artificial stimulation. All the Bantu mothers had had more pregancies than the Europeans but this was not thought likely to have any effect on the results.

Each series was divided into two categories according to whether, in addition to their 7S antibodies, 19S antibodies were present or absent:

(1) no 19S antibodies, or 19S antibodies to a titre of 2, and

(2) 19S antibodies to a titre of 4 or more.

Those with 19S antibodies to a titre of 2, were included with those without because a high titred 7S antibody in the presence of its own serum will sometimes cause weak visible agglutination which disappears as soon as the serum is diluted.

TABLE 1

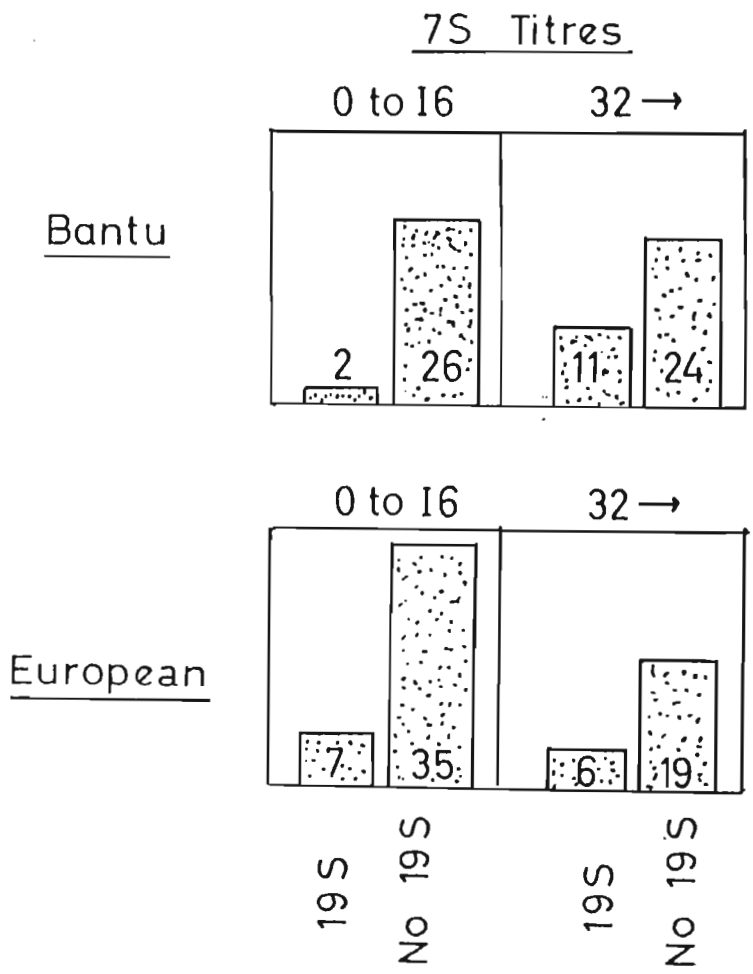
The initial results were disappointing. Approximately 80% of both Bantu and European women qualified for category (1) and 20% for category (2). The observed difference could not therefore be explained this way. The two categories were now subdivided to find the incidence of 19S antibodies in relation to the **titres** of the 7S antibodies.

TABLE 2

This time the difference was more obvious. There were a greater number of Europeans than Bantu with 19S antibodies when the 7S titre was less than 32, and more Bantu than Europeans when the 7S titre was 32 or

TABLE 2

I9S Antibodies in Relation to 7S Antibody Titre



higher. In category (1), that is those without 19S antibodies, the number of Bantu was slightly above that of Europeans with 7S titres of 32 and higher but fewer Bantu had 7S titres of less than 32.

Taking their group alone, Bantu with 19S and 7S titres of 32 and above were in excess of those with 19S titres and 7S titres below 32, while the numbers without 19S titres were approximately the same for each group. Europeans with 19S antibodies, however, were almost equal in number regardless of the 7S titre, and in those without 19S antibodies the greater proportion had 7S titres below 32. Explained in simpler terms 19S antibodies occured more frequently in the Bantu women when the 7S titre was high than when it was low, whereas the proportion in the European women was about equal in both.

There are several possible explanations. 19S antibodies may persist longer in Bantu than in European women; or Bantu women may produce higher 7S titres earlier, while 19S antibodies are still present. Also some Bantu red cell antigens are known to be stronger than their European counterparts, and it may be that the response of Bantu women to Bantu antigens differs from that of European women to European antigens.

Murray[2] observed that 19S antibodies could reappear during subsequent pregnancies, particularly those ending in stillbirth. This in itself might be enough to account for the increased incidence in the Bantu, since in our series they had all more pregnancies than the Europeans, providing that it could be shown that the greater the number of pregnancies the greater the chance of the persistence of 19S antibodies.

The high incidence of 19S antibodies in the Bantu was an unusual finding that needs explanation but it is appreciated that the number of cases investigated was very small and the results were not statistically significant. A much larger number will be studied as soon as the material becomes available. The significance of 19S antibodies may perhaps be different in Bantu and Europeans. At this stage it appears that a new assessment of the criteria for the diagnosis of cases of haemolytic disease of the newborn may be needed and a new set may have to be created especially for use in Bantu populations.

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Paper 15

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A Screening Test for Antibodies

Using 2-Mercaptoethanol

P. Moores *and* B. G. Grobbelaar
The Natal Blood Transfusion Service, P.O. Box 2356, *Durban, South Africa*

Abstract

The activity of 19S antibodies is destroyed by the addition of 2-mercaptoethanol. A screening test employing this substance for the selective detection of 7S blood group antibodies in pregnancy is described.

The results of testing 20,273 antenatal blood samples using this test in parallel with our routine serological procedures are reported.

Every year our organization examines some 30,000 blood samples from pregnant women for antibodies that may cause haemolytic disease of the newborn. In addition to the customary ABO and Rhesus typing of the blood samples, the serum is screened for antibodies by a bromelin technique using cells specially selected to contain the common blood factors. If the bromelin screening test is positive, or the specimen is Rhesus negative, or the history is suggestive of possible maternal iso-immunization (stillbirth, jaundiced infants, previous blood transfusions, etc.), an indirect antiglobulin test is also performed. Whenever the bromelin screening test is positive, we attempt to identify the antibody, using a selected panel of test cells. In our experience the vast majority of antibodies detected by the bromelin technique are negative by the indirect antiglobulin test and do not cause haemolytic disease of the newborn.

It is generally accepted that 19S antibodies do not cross the placental barrier and therefore do not cause haemolytic disease of the newborn. It would therefore seem logical to determine whether an antibody is of the 7S or 19S variety, when detected during the antenatal period.

There are several known methods of differentiating between 19S and 7S gamma globulin,[1, 3] all fairly time-consuming. One of them uses the sulphydryl compound 2-mercaptoethanol, which is capable of splitting disulphide bonds, causing dissociation of 19S but not of 7S gamma globulin. It is claimed that the 2-mercaptoethanol requires removal by dialysis or vacuum extraction before the treated sample can be used.[1, 3] However, Watson[5] indicated that its presence does not interfere with the antiglobulin reaction. His observation led us to apply a simple and rapid technique, using 2-mercaptoethanol, to differentiate between 7S and 19S blood group antibodies.

Materials and Methods

20,273 specimens submitted for routine antenatal investigation were subjected to the 2-mercaptoethanol and the bromelin test in parallel in our routine procedure as described in the opening paragraph.

The 2-mercaptoethanol used throughout was supplied by Fluka Ag. Chemische Fabrik Buchs SG, Switzerland. A concentration of 0.1 molar in 0.89% saline was selected for standard use and was obtained by adding 0.56 ml. 2-mercaptoethanol to 99.44 ml. saline. If the solution is kept out of direct sunlight in a well-stoppered brown glass bottle, there is little loss of reactivity over three weeks.

The 2-Mercaptoethanol and the Bromelin Screening Test. To one volume of the patient's serum is added one volume of 0.1 molar 2-mercaptoethanol. As a control, one volume of saline is substituted for the 2-mercaptoethanol in another test tube. After mixing, both tubes are placed in an incubator at 37° C. for one hour. One volume of a 2% suspension of bromelin-treated test cells is then added to both test and control tubes. The bromelin treatment consisted of prior incubation with an equal volume of 0.5% bromelin solution for 10 minutes at 37° C., followed by 3 saline washes. After mixing, the tubes are allowed to stand at room temperature for one hour before being read macroscopically over a light box.

A pure 19S anti-H serum and a weak 7S anti-Rh_0 (anti-D) are used as controls, and the 2-mercaptoethanol is considered to be unsuitable unless it completely inactivates the anti-H but has no effect on the anti-Rh_0 (anti-D).

The red cells used to detect antibodies were from a pool of donations known to contain between them all the principle antigens of the MNSs, P, Rhesus, Lutheran, Kell, Lewis, Duffy and Kidd blood group systems. They were preserved in buffered glycerol in tripotassium citrate solution,[4] and stored frozen at −20° C. Small samples were thawed daily and deglycerolyzed by dialysis against 0.89% saline using Visking tubing.[6]

Results

The bromelin screening test detected 3,226 antibodies (Table 1). By contrast the 2-mercaptoethanol and the bromelin screening test detected only 524, which represents a reduction from 15.91% to 2.58%.

Table 1: Comparison of the Proportion of Positives Found in Testing 20,273 Antenatal Specimens for Blood Group Antibodies Using Two Different Screening Test Techniques

Screening Test Technique	*Number of Specimens*	
	Positive	*Negative*
Bromelin	3,226 (15·91%)	17,047
2-mercaptoethanol + Bromelin	524 (2·58%)	19,749

All equivocal and weakly positive results were in the first instance regarded as positive. With the 2-mercaptoethanol testing system there is some inclination to rouleaux formation, and to eliminate apparent positives

caused by this, the test was repeated, the results being read microscopically. Of the original 524, only 232 (1.41%) were finally found to be positive.

The 232 antibodies were investigated in greater detail to determine their specificities (Table 2). They were found to consist of 127 (54.7%) that were clinically significant and 105 (45.3%) which, although of the 7S variety, showed themselves to be not clinically significant as they were specific and non-specific cold antibodies.

Table 2: Identification of 232 Antibodies which were Positive with the 2-Mercaptoethanol Screen Test

Clinically Significant		*Not Clinically Significant*	
Specificity	*Number Detected*	*Specificity*	*Number Detected*
Anti-D	102	Anti-P	8
Anti-E	9	Anti-P + cold auto	2
AntiE + c	1	Anti-P + Le^a	1
Anti-E + c + K	1	Anti-P + Le^a + Le^b	1
Anti-$\bar{c}$	4	Anti-Le^a	12
Anti-$\bar{e}$	3	Anti-Le^a + Le^b	6
Anti-C	2	Anti-Le^a + cold auto	9
Anti-C + K	1	Anti-Le^a + cold saline	2
Anti-K + cold auto	1	Anti-Le^b	1
Anti-K + Fy^a	1	Anti-Le^b + cold auto	1
Anti-K	1	Anti-S	1
Unidentified	1	Cold saline antibodies	12
		Warm auto antibodies (haemolytic anaemia)	2
		Cold auto antibodies	26
		Cold Saline + cold auto	1
		Contaminated specimens	2
		No detectable antibodies	18
Total	127 (54·7%)	*Total*	105 (45·3%)

All samples positive with the bromelin screen test but negative with the 2-mercaptoethanol screen test were shown to consist also of specific and non-specific cold antibodies. Most of them were anti-Le^a, anti-P and cold auto-antibodies. There was one exception, an anti-rh″ (anti-E), for which no positive indirect antiglobulin test could be demonstrated and which was considered to be an example of a naturally occurring Rhesus antibody. Confirmatory steps showed that this antibody was indeed destroyed by 2-mercaptoethanol, and the pregnancy concluded with a normal, unaffected infant.

Discussion

This simplified 2-mercaptoethanol test is of considerable value in detecting those antibodies which may cause haemolytic disease of the newborn, as all 19S antibodies will give a negative result. As the study has clearly shown, however, all 7S antibodies are not necessarily of clinical significance,

because the specificity and the thermal amplitude of the antibody also have a bearing on the ability of the antibody to cause foetal red cell destruction.

The main advantage of this screening system is that it obviates the necessity of carrying out laborious identification procedures on the many antibodies which will be detected by using an enzyme technique as the basic antibody screening test. Clearly the same objective can be achieved by using the indirect Coombs test as the basic screening technique, but this would be laborious and expensive if large numbers of specimens have to be investigated.

A valid criticism of this screening procedure, which would also apply to a screening procedure employing the indirect antiglobulin test, is that it will not detect many antibodies which, although not of clinical significance, may be of academic interest. However, it is a matter for every laboratory to consider the extent to which it is able to increase its costs with a view to detecting and studying antibodies which are not of immediate clinical significance.

It could be argued that the addition of 2-mercaptoethanol has a diluting effect which will cause very weak antibodies to go undetected. It is debatable whether an antibody which is so weak that it cannot be detected in a dilution of 1 or 2 is, in fact, of clinical significance. Nevertheless, this should be borne in mind.

Our experience with 2-mercaptoethanol confirms Watson's observation[5] that its presence does not interfere with the agglutination reactions, and that there is no need to remove it by dialysis after treatment of the serum. This greatly simplifies the application of the technique in blood group serology.

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Paper 18

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SEPARATUM

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Haemolytic Disease of the Newborn due to ABO Incompatibility

A New Aid to Diagnosis

P. MOORES, B. G. GROBBELAAR and V. WARD

Natal Institute of Immunology, Durban

KOCHWA *et al.* [2] found that certain ABO isoagglutinins, after separation with the bulk of 7 S γ-globulins from other serum proteins by anion exchange chromatography, resisted inhibition by specific soluble blood group substance (hog and horse). In a group of 53 type O mothers they found 5 with such resistant isoagglutinins. Four of these mothers gave birth to type O babies and the fifth a type B child which required an exchange transfusion for haemolytic disease of the newborn.

ABO incompatible babies of mothers whose isoagglutinins were readily inhibited showed no clinical evidence of haemolytic disease. Mothers with a history of severe ABO haemolytic disease and donors immunised with specific soluble blood group substances, however, all had noninhibitable isoagglutinins.

The technique of KOCHWA *et al.* [2] was suitable for antenatal specimens, but the two day delay required for anion exchange chromatography prevented rapid diagnosis in the neonatal period. This paper reports that, by using 2-mercaptoethanol [4] and modifying the concentration of the serum, the delay can be reduced to a few hours.

Materials and Methods

Three series of specimens were used for the test:

1. Antenatal samples of clotted blood from mothers (of all ABO types) with a poor obstetric history (miscarriages, abortions, stillbirths, neonatal jaundice, neonatal deaths and previous blood transfusions).

2. Antenatal specimens selected because the results of tests on their infants' cord blood at the time of delivery were available. Every effort was made to avoid selection on the basis

of the obstetric history in these cases, but medical practitioners, for obvious reasons, more often send in cord blood samples from the infants of such mothers.

3. Unselected maternal blood that had been investigated for the first time at delivery only. No antenatal results were available.

Samples containing antibodies other than A and B isoagglutinins were excluded. 2-Mercaptoethanol[1] which causes the dissociation of 19 S antibodies but has no effect on 7 S antibodies, was used to eliminate 19 S isoagglutinins from each maternal sample. At a 0.1 molar concentration in saline (prepared by adding 0.56 ml to 94.4 ml of 0.89% saline) 2-mercaptoethanol showed no deterioration for up to 3 weeks at room temperature tightly stopped in a brown glass bottle.

To inhibit the maternal 7 S isoagglutinins, as specific soluble blood group substances were difficult to obtain, samples of saliva (collected from A and B secretors) were boiled for 20 min in a water bath. After removal of the deposit by centrifuging, the A and the B samples of saliva were pooled separately. A convenient dilution of 1:100 in saline was selected for use confidently since tests showed that inhibition could be detected at a dilution of 1:32,000.

Suspensions of enzyme-treated red cells of type A_1 and type B were used for the test. After being washed twice in saline, the cells were packed and treated by adding an equal volume of 0.5% bromelin solution [3] for 10 min at 37°C followed by 3 saline washes. A 2% suspension of these 'bromelin-treated' cells was prepared for use.

The technique for the detection of non-inhibitible isoagglutinins is as follows: One volume of serum was mixed with one volume of 0.1 molar 2-mercaptoethanol and incubated at 37°C for one hour. Fourteen volumes of saline were then added to adjust the dilution to 1:16. One volume of the 1:16 dilution was placed in each of 2 test tubes marked 'A' and 'B' respectively. To the 'A' tube one volume of 1:100 A secretor saliva was added and to the 'B' tube one volume of 1:100 B secretor saliva. Both tubes were left at room temperature (±20°C) for 30 min, after which one volume of a 2% suspension of bromelin-treated A_1 cells was added to the 'A' tube and of B cells to the 'B' tube. After incubation at room temperature for 1 hour the results were read by lightly tapping the tubes over a Diamond light-box, without prior centrifuging. Tests were positive when either the 'A' or the 'B' tube or both showed obvious macroscopic cell clumping.

The minimum time required to obtain a result was 2 ½ hours.

Results

In the first series (600 maternal specimens) 87 (14.5%) had non-inhibitible isoagglutinins. There were only 44 cord blood samples in this group but, as the medical practitioners had been asked particularly in each case to watch for jaundice, it is more than likely that all the affected infants were included. Of these 44, 17 (2.8%) by clinical or serological standards, were suffering from haemolytic disease of the newborn; 18 were not affected though the mother/child ABO types were not homologous; and 9 had homologous ABO types.

In the second series (500 maternal specimens) 90 (18%) had non-inhibitible isoagglutinins. The results of tests on all the infants were

[1] The sample used was supplied by Fluka AG Chemische Fabrik Buchs, SG, Switzerland, and was marked 'Purum'.

Table I. Results obtained in an investigation for the detection of non-inhibitible isoagglutinins in maternal sera

Series	Origin	Non-inhibitible isoagglutinins			Inhibitible agglutinins		
		No.	Cases with haemolytic disease of the newborn		No.	Cases with haemolytic disease of the newborn	
			No.	percentage		No.[1]	percentage
53 Group O only	Kochwa *et al.* [2]	4	1	25	49	0	0
600 All ABO groups	Poor obstetric history (series I)	87	17	19.5	513	0	0
500 All ABO groups	Availability of infant cord blood (series II)	90	18	20	410	1	0.25
115 All ABO groups	Tested at time of delivery only (series III)	15	2	13.3	100	0	0

[1] Since cord blood samples were received from only a proportion of the cases, these figures must be taken to indicate the number of infants whose condition justified serological investigation.

available; 18 (3.6%) suffered from haemolytic disease of the newborn; 23 were not affected though the mother/child ABO types were not homologous and 49 had homologous ABO types. One infant was affected although the maternal isolaggutinins had been inhibitible 2 months before. It is possible that at that stage the maternal isoagglutinins were 19 S and 7 S isoagglutinins developed later.

The third series (115 unselected maternal and cord blood specimens) contained 15 (13%) with non-inhibitible isoagglutinins; 2 (1.7%) of the infants suffered from haemolytic disease of the newborn.

Discussion

The reasons for the various steps of the technique have been tabulated.

1. 2-Mercaptoethanol at a 0.1 molar concentration in saline has the advantage that it can be added directly to serum to dissociate the

19 S isoagglutinins and does not need to be removed subsequently. 19 S antibodies do not pass the placental barrier and are therefore unlikely to be implicated in haemolytic disease of the newborn. 2-Mercaptoethanol is also easy to use and is rapid in its action.

2. After the removal of the 19 S isoagglutinins, the heated samples are diluted with saline to 1:16. GROBBELAAR and GORDON [1] showed that 7 S isoagglutinins with a titre of less than 1:32 are found commonly in human serum and are of no significance to the infant. Dilution at this stage ensures that at the time the red cell suspension is added, the final dilution will be 1:32.

3. The samples are divided into 2 parts (one for the addition of A-secretor saliva and the other for B-secretor saliva) to investigate each isoagglutinin separately. The success or otherwise of inhibition is measured by adding a suspension of enzyme-treated cells.

4. Enzyme treatment of the red cells renders them agglutinable by 7 S isoagglutinins. In the test, prior treatment rather than simple addition of enzyme to the test tubes removes the possibility of additional dilution of the serum beyond 1:32. No studies by an indirect Coombs test have been done, as the results were not found to be reliable.

KOCHWA *et al.* [2] detected one case of haemolytic disease in 4 mothers found to have non-inhibitable isoagglutinins. In our first 2 series the incidence was 1:5, and 1:7 in the third series.

Since it is very probable that, regardless of cause, most infants with more than a 'normal' amount of jaundice in the neonatal period will undergo clinical and serological investigation, the figures in our 3 series can be taken to represent the maximum number of truly affected cases. The one affected infant born to a mother whose isoagglutinins had previously been inhibitible supports this view.

Our results show that infants affected by haemolytic disease due to ABO incompatibility are most likely to be the offspring of mothers whose isoagglutinins are non-inhibitible. This supposition is in agreement with the findings of KOCHWA *et al.* Moreover, modifications of technique as set out in this paper have not detracted from the value of the test and have led to an increase in the speed with which the results can be obtained.

Summary

Modifications made to the original method of KOCHWA *et al.* for the detection of non-inhibitible A and B isoagglutinins in pregnant mothers permit results to be obtained rapidly

and easily. This makes the technique very much more useful in the diagnosis of ABO haemolytic disease of the newborn, both in the neonatal period and antenatally. Results are presented which agree with the findings of KOCHWA *et al.* that most ABO-affected infants are born to mothers with non-inhibitible isoagglutinins.

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Authors' address: Miss P. MOORES, Dr. B. G. GROBBELAAR and Mrs V. WARD, The Natal Blood Transfusion Service, 149 Prince Street, *Durban* (South Africa).

Paper 17

Some Problems of Rh Grouping by the Chown Technique.

B. Dobie and P. Moores.
Natal Institute of Immunology,
Durban.

In 1944, Dr. Bruce Chown[1] introduced a new technique for Rhesus grouping large numbers of blood samples with a very small amount of 19S(M) anti-D serum which became known as the 'Chown' test. In it the anti-D serum was taken up in a capillary tube 90mm x 0.4mm, followed immediately by an equal volume of a 20-30% suspension of the patient's washed red cells in saline. After inversion the capillary was placed a little above a glass panel at an angle of 45°, illuminated from below by an electric light bulb. Readings were made from 5 minutes to a maximum of 60 minutes after commencement. A granular appearance of the red cells indicated a positive result and absence of granulation a negative result.

The 'Chown' test has been in use in our laboratory for a number of years for the Rhesus grouping of antenatal blood specimens. All 'Chown' negatives were tested with enzyme — and antiglobulin — reacting 7S (γG) anti-D sera and any positive by these techniques were labelled subtype D^u. In spite of an expected high incidence of D^u in Bantu, however, the proportion detected seemed rather too high and in samples from certain remote areas D^u samples were often predominant. An investigation was commenced and the results will be reported in this paper.

MATERIALS AND METHODS

For a period of 4 weeks all "D^u" blood samples were subjected to the following tests, all performed by the same person to eliminate slight variations of technique.

a) a repeat of the 'Chown' test with each of three standardised chown anti-D sera from different immunised mothers. The technique was followed closely, with careful timing, temperature control and attention to the angle of the glass panel in a specially made 'Chown' light box.

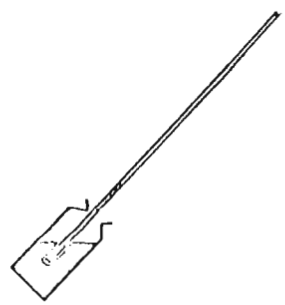
b) Simple tube tests with the same 3 chown antisera which made it possible to examine the cells for clumping under the microscope as well.

c) a tube test using a potent saline — agglutinating anti-D serum not standardised for the 'Chown' technique.

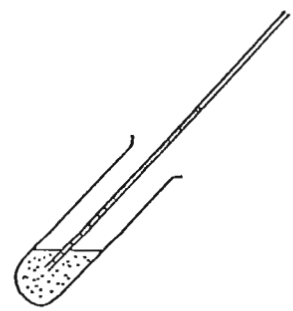
d) tube tests using an enzyme and an antiglobulin reacting serum, but omitting the enzyme or the antiglobulin respectively. Negative results in this test ensured that the samples were not agglutinating spontaneously for any reason.

e) an enzyme and an antiglobulin test in which the enzyme or the antiglobulin were added.

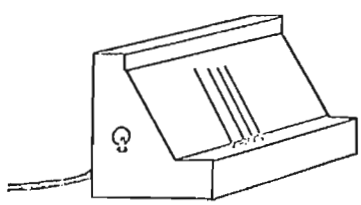
Capillary Tube.



Taking up anti-D



followed by 20% Red Cell Suspension



and placed on a Chown box.

This test determined that the samples had the D^u antigen.

In addition all the samples were stored at 4°C, and were retested by the foregoing methods on each of 2 subsequent days to assess the effects of passage of time on the strength of the "D^u" antigen.

RESULTS AND DISCUSSION

The overall incidence of "D^u" samples was 3.8% of all antenatal specimens, or 2.5% in Europeans, 2.1% in Asians and 7.4% in Bantu. The expected incidence was less than 1% in Europeans, and 6% in Bantu[1]. In their book entitled 'Heredity of the Blood Groups', Wiener and Wexler define 'D^u', or Rh_o, as blood which

TABLE 1

Chown Test			Tube Test with Chown Serum			Saline-aggl. anti-D	CONTROLS				Nos. detected
1	2	3	1	2	3	(potent)	Enzyme anti-D without enzyme	Antiglob. anti-D without antiglobulin	Enzyme. anti-D with enzyme	Antiglob. anti-D with antiglobulin	
+	+	+	+	+	+	+	–	–	+	+	3
+	+	+	+	+	–	+	–	–	+	+	1
+	–	+	+	+	+	+	–	–	+	+	1
+	–	+	–	+	+	+	–	–	+	+	2
+	–	+	–	–	+	+	–	–	+	+	2
+	–	–	+	–	–	+	–	–	+	+	1
–	+	–	–	+	–	+	–	–	+	+	1
–	–	–	–	–	–	+	–	–	+	+	3
–	–	–	–	–	–	–	–	–	+	+	5

"fails to react even when using potent saline anti-Rh_o (Anti-D) serum", but which is agglutinated by some but not all enzyme — or antiglobulin reacting anti-Rh_o (anti-D) sera. From the results of the investigation (Table I) we observed that the majority of those specimens which we had been calling 'D^u' were being agglutinated by saline – agglutinating anti-D sera and were, therefore, not true D^us. They were, however, generally weaker than the Rhesus positive controls, as expected, and were agglutinated sometimes by one, sometimes by another of the 'chown' anti-Ds. Without exception all were agglutinated by the potent saline – agglutinating anti-D serum used in a tube test.

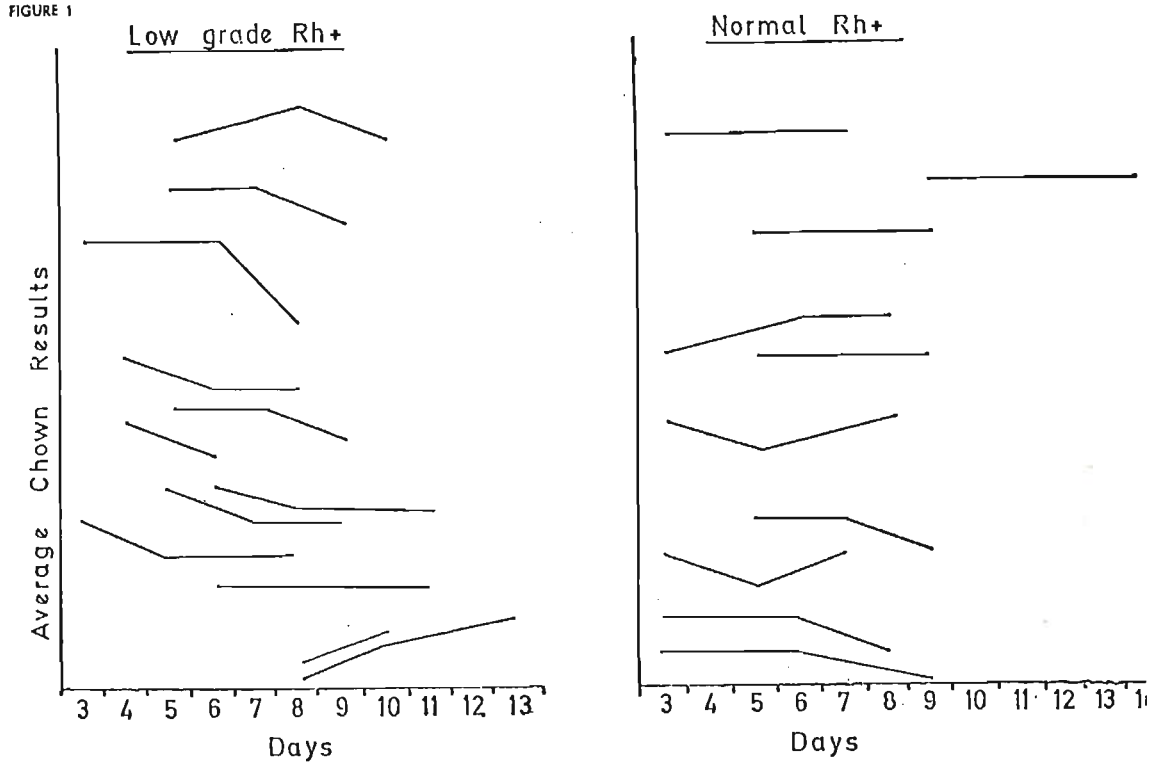
In using the 'chown' technique it was plain that many low grade Rhesus positive persons were being called D^u incorrectly. This was an error on the right side, fortunately, though it increased the labour in the laboratory which must follow up with additional group and antibody tests, and might give a false impression of the proportion of mothers whose unborn infants were at risk of haemolytic disease.

Figure I shows that over a period of some days most of the so-called 'D^u' samples deteriorated in strength, measured by 'Chown' test values, whereas most of the normal Rhesus positive samples did not show this tendency. The high incidence of D^u persons living in certain outlying areas was, therefore, in our opinion of the results obtained, due to a rather more rapid deterioration of the Rhesus antigen of low grade Rhesus positive blood samples than of normal samples. It is possible to imagine that a similar condition might arise if the samples were subjected to a long period of unrefridgerated travel before being tested.

CONCLUSIONS

Figure II is a diagram drawn in an attempt to represent the types of Rhesus positive antigen. The horizontal subdivisions are the various techniques. It shows that, theoretically, a great many variations are possible depending on the amount of antigen which may be present. If this is true, then Rhesus grouping techniques may not be detecting different specific genes. The distinctions may be being made by anti-

FIGURE 1



serum capability and will, therefore, vary according to the antiserum used.

SUMMARY

A number of D^u blood samples, negative by chown test but positive with an enzyme or an antiglobulin type anti-D serum, were examined by various Rhesus grouping techniques and types of anti-D sera and were found to consist of mainly low grade Rhesus positive samples.

When stored at 4°C for a number of days, the strength of the D antigen appeared to deteriorate compared with normal Rhesus D positive samples. This effect may account for the preponderance of D^u samples received from certain outlying areas which have had several days of unrefrigerated travel.

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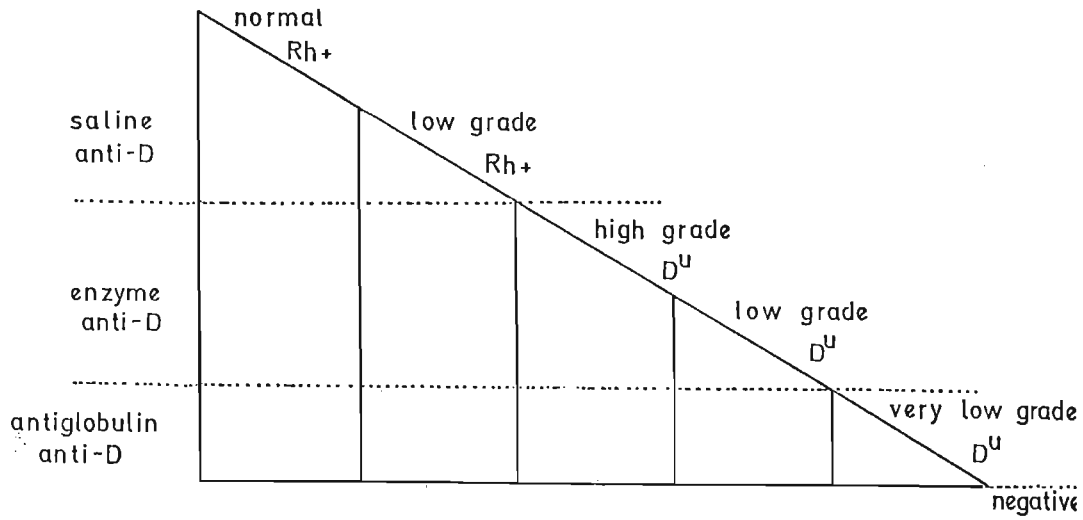

FIGURE 2 Rhesus D Antigen.

CHAPTER XII

BLOOD GROUP PHENOTYPE AND GENE FREQUENCIES IN BLACK, WHITE, INDIAN AND COLOURED BLOOD DONORS OF NATAL

XII.1 THESES AND PAPERS

Tables XII.1 to XII.33 contain the results of the author's blood group frequency studies in Natal blood donors. The data were obtained from her M.Sc. and Ph.D. theses, from Paper 14 and Paper 54.

XII.2 INTRODUCTION

Very few earlier studies in the Black, White, Indian and Coloured populations of Natal were available when the author wrote her M.Sc. and Ph.D. theses [Table XII.1]. Nurse, Weiner and Jenkins [1985] have since added to the frequencies in the Natal Black population (Zulus). The results of the earlier studies are in the Appendix.

The blood samples in this study were from Black, White, Indian and Coloured blood donors of the Natal Blood Transfusion Service. The Black donors were mainly Zulus and the White donors principally from Western Europe. The Indian donors, included in this thesis both as a single population and as separate populations who spoke either Tamil, Telegu or Hindi at home or who were Moslems, were the descendants of Indians from India. Their ancestors had been imported into Natal from the year 1860 onwards to work as indentured labourers on the sugar plantations. The Indian names of the Indians were used to assess the language spoken by each donor at home and the religion each professed; educated Indian colleagues kindly gave their advice with this. Historical and other records suggested that the Hindi-speakers had come from the eastern part of northern India, the Telegu-speakers from the eastern part of central India and the Tamil-speakers from southern India. Since converts to Islam often adopt Moslem names, the Moslem donor population had to be considered largely amorphous. The Coloured

donors were of local mixed ethnic origin. As far as possible, random blood group and antigen distribution was assured by including only males and unmarried females who had not previously donated blood.

The results in Tables XII.2 to XII.33 are of phenotype, haplotypes, genes, alleles and gene complexes in the ABO, MNSs, P, Rh, Lutheran, Kell, Lewis and Secretor, Duffy, Kidd, Colton, Diego and In^a systems and of the antigens Xg^a, Sd^a and I. They include those in the author's M.Sc. and Ph.D. theses and those in paper 14 and paper 54. In each blood group system, the frequencies estimated for the four populations are presented in parallel.

The studies were all made for the author's own interest and to provide a record of the blood group frequencies for the benefit of future blood bank and other workers. Some frequencies were useful to the Natal Blood Transfusion Service, whose donors are bled conservatively. The Service also maintains a list of donors with rare groups and their relatives. The phenotype and allele frequencies, and the χ^2 calculations for goodness of fit, have all been re-confirmed and, where necessary, amended.

XII.3 COMMENTARY

XII.3.1 Attainments in Durban, 1961 to 1991

XII.3.1.1 ABO system

Tables XII.2 and XII.3 showed that the highest *B* (*q*) allele frequency occurred in the Indian donors. The greatest contribution towards this had been made by the Hindi-speaking Indians. In general, the frequencies in the Indians were similar to those recorded in Indians elsewhere [Mourant, Kopeć and Domaniewska-Sobczak, 1976]. The White donors had high *A* (*p*) and *O* (*r*) allele frequencies; in this they were similar to Whites in Western Europe [Mourant et al., 1976]. The results in Tables XII.4a and XII.4b confirmed that the Black donors had the highest A_{bantu} and $A_{bantu}B$ phenotype frequencies. The 4% A_{bantu} phenotype frequency in the group A Blacks accorded with the earlier findings of Brain [1966]. The unusually high $A_{bantu}B$

phenotype frequency in the Blacks (15,16%) was almost certainly due to the inclusion of some donors with A^2B genes. As the product of the *A* gene may be depressed when a *B* gene is present [Issitt, 1985, p151], these genotypes are usually confirmed by family studies. Table XII.5 showed that the H antigen of the group AB Indians and Blacks varied considerably in strength. H antigen was also detected in some donors with group A_1B and not in other donors with group A_2B red cells. This was surprisingly in converse to the expected position.

XII.3.1.2 MNSs system.

Table XII.6 contained the results with anti-M and anti-N. It revealed that the Indians had the highest *M* and the Whites the highest *N* allele frequencies. When anti-S was included in the studies, Table XII.7 showed that the Whites had the highest *Ns* allele frequency. The χ^2 calculations for the investigations in which anti-s was also included indicated, although the results for the Indians were satisfactory, that those for the Coloureds were far less so. The easiest answer was that the red cells of some type MSs Coloureds had given s- instead of s+ results. As the Coloured donor studies had taken several years to complete, it had not been possible to test them all with the same batch of anti-s. The problem emphasised the importance of calculating χ^2, for it may be the only means by which a set of results is confirmed to be internally consistent [Race and Sanger, 1968, p14]. The S- donors' red cells had all been tested in parallel with anti-U (this specificity reacts with both S+ and s+ red cells), but no similar method for confirming the presence of s in S+ red cells was known.

The results of the M_1 antigen frequency study in the Indian donors were presented in Table XII.9. M_1 is often identified in African Blacks [Race and Sanger, 1968, p107], but the Natal Black donors were not tested for this antigen, as suitable anti-M_1 reagents became available only after the work had been completed. The results of the study with anti-Henshaw in Table XII.10 showed that the *He* frequency in the Black donors was approximately 4%. This gene was associated in them

predominantly but not exclusively with *MS*.

XII.3.1.3 P system

Table XII.11 contained the results with the donors' red cells and anti-P_1. The findings in the Black donors accorded with the known exceptionally high P_1 frequency reported in Black populations elsewhere [Mourant et al., 1976, p341-342]. They were also in agreement with the frequencies in the other Southern African Black populations recorded by Nurse et al., [1985, p310-311].

XII.3.1.4 Rh system

The frequencies in the Rh system determined with anti-D, and by testing all the D- blood samples with anti-CD, anti-E and for D^u confirmed (Table XII.12) that the highest dce phenotype frequency occurred in the White donors. The D^u phenotype was identified in all three populations tested: as expected, it occurred most often in the Black donors. The Blacks had the highest dCe and the Whites the highest dcE phenotype frequencies. The term D^u was used for those red cells which gave negative results in tests with saline anti-D reagents after the tests had been incubated at 37°C, centrifuged lightly and read both macro- and microscopically. Some but not all the D^u gave weaker positive results than the D+ red cells in tests with enzyme- and indirect antiglobulin- reacting anti-D reagents. Table XII.13 records the Rh phenotypes determined with anti-D, anti-C, anti-E, anti-c and anti-e and by testing the D- red cells for D^u. The haplotype frequency calculations showed that the Indian donors had the highest *DCe*, the White donors the highest *dce*, *DcE* and *dcE* and the Black donors the highest *Dce* frequencies.

Table XII.14 confirmed that C^w was closely associated with the haplotype *DCe* in both the White and Indian donors. Table XII.15 recorded the results with the Black donors' red cells and anti-hr^s. The hr^s- phenotype was confirmed to be closely associated with *Dce* and *DcE* haplotypes.

XII.3.1.5 Lutheran and Kell systems

Table XII.16 contained the results with the donors' red cells and anti-Lu^a. The results showed that the *Lu^a* frequency in the Black donors and the White donors was approximately equal [Lu(a+) red cells were not identified in the Indian donors]. Tables XII.17 and XII.18 showed, as expected, that the Kell antigens K and Kp^a occurred predominantly in Whites.

XII.3.1.6 Lewis and Secretor systems

In the Black donors and the White donors, Table XII.19 indicated that the secretor genes *Se* and *se* were distributed more or less equally. Approximately 76% of the Black donors secreted Le^a substance [Table XII.20]. Among the 171 Blacks in Table XII.21, the *Les* : *les* ratio in those with Le(a-b-) red cells was approximately 4 to 1 [paper 14]. The Black donors who did not secrete Lewis substances evidently had true Le(a-b-) red cells. Among the 163 Black donors in Table XII.21, all eight with Le(a-b-) red cells secreted Le^a substance. This suggested that they belonged with the 31 donors who had Le(a+b-) red cells. The number of donors with Le(a-b-) red cells who did not secrete Lewis substances was nonetheless surprisingly small.

In the tests with the donors' red cells and anti-Le^{bL} (anti-Le^{bH} was not used), the highest Le(a-b-) phenotype frequency occurred in the Black donors (Table XII.20). Tables XII.21 to XII.23 showed that the Black, White and Indian donors had approximately equal frequencies of Le(a+) red cells (22% and 19%, 23% and 24%, respectively). Among the Indians, the Tamil-speakers had the highest Le(a+) phenotype frequency. The presence of Le(a+b+) red cells confirmed the earlier suggestion of Mourant et al. [1976, p56] that our current belief about *Le/le* gene inheritance may not be complete.

XII.3.1.7 Duffy system

In Tables XII.24 and XII.25, the Fy(a+) phenotype and *Fy^a* gene

frequencies were seen to be highest in the Indian and lowest in the Black donors. The results in Table XII.25 confirmed that the low frequency in the Blacks was due to the large number with Fy(a-b-) red cells. As the Fy(a-b-) phenotype is malaria-associated [Issitt, 1985, p283-284] and many Natal Blacks live in the northern part of Natal (Zululand) where malaria is endemic, the low Fy(a+) frequency in the Blacks had been expected.

XII.3.1.8 Kidd, Colton, Diego and In systems and Xg^a antigen

The higher Jk^a than Jk^b frequency in the Indian donors was evident in Table XII.26. Table XII.27 revealed that the Xg(a+) frequency was higher in the Indian male than the Black male donors. Tables XII.28, XII.29 and XII.30 showed that 1-2% of the Indian donors had Co(a+), Di(a+) and In(a+) red cells in the Colton, Diego and In^a systems, respectively.

XII.3.1.9 Sd^a (Sid) and I antigens

Table XII.31 indicated that Sd(a-) red cells occurred more frequently in the Indian and Black than in the White and Coloured donors. Table XII.32 showed that the Sd(a+++) phenotype was present in the Indian donors. As polyagglutination was not detected, their cells were not Cad+. In the tests with anti-I, Table XII.33 revealed that the Black donors had a low I- phenotype frequency.

Table XII.1 Previous studies in populations of Natal and Zululand

Year	Author	Population	Reference
ABO system			
1936	Elsdon-Dew	Zulus	Thesis No.39, S.A.Institute for Medical Research VII, p217-300.
1955	Zoutendyk	Zulus	In Mourant, Kopec and Domaniewska-Sobczak. The ABO blood groups - comprehensive tables and maps of world distribution. Blackwell, Oxford, 1958.
1958	Hirsch	Africans, Coloureds and Whites	Transactions of the Royal Society of Tropical Medicine and Hygene 52, 408-410.
1960	N.B.T.S.	Blacks	Natal Blood Transfusion Service, unpublished data.
1961	Buckwalter, Kark and Knowler	Bantu	Archives of Internal Medicine 107, 558-567.
1966	Brain	Bantu	Vox Sanguinis 11, 686-698.
Rh system			
1951a	Shapiro	Zulus	South African Medical Journal 25, 951-955.
1958	Hirsch	Blacks, Indians and Whites	Transactions of the Royal Society of Tropical Medicine and Hygene 52, 408-410.

Table XII.2 ABO system

Donors tested with anti-A, -B and -A,B

Donors	Number tested	Phenotypes A No.	%	B No.	%	O No.	%	AB No.	%
Blacks	8281	2499	(30,18)	1607	(19,40)	3766	(45,48)	409	(4,94)
Whites *	3964	1532	(38,64)	403	(10,17)	1882	(47,48)	147	(3,71)
Indians *	2720	594	(21,84)	867	(31,87)	1051	(38,64)	208	(7,65)
Tamil	1211	256	(21,14)	371	(30,64)	510	(42,11)	74	(6,11)
Telegu	493	97	(19,67)	158	(32,05)	199	(40,37)	39	(7,91)
Hindi	644	151	(23,45)	220	(34,16)	217	(33,70)	56	(8,69)
Moslem	372	86	(23,12)	114	(30,64)	143	(38,44)	29	(7,80)

* = Total Tamil + Telegu + Hindi + Moslem

Donors	Allele frequencies p	q	r	X^2 for ABs	p
Blacks	0,1946	0,1302	0,6752	0,37	0,7
Whites *	0,2405	0,0719	0,6876	1,04	0,5
Indians *	0,1599	0,2218	0,6183	1,88	0,2
Tamil	0,1470	0,2046	0,6484	0,03	0,9
Telegu	0,1483	0,2241	0,6276	2,02	0,2
Hindi	0,1762	0,2439	0,5799	0,01	0,9
Moslem	0,1685	0,2149	0,6166	0,25	0,7

Allele frequencies calculated according to the formulae of Bernstein quoted by Race and Sanger, 1968, p14.

X^2 calculated according to Fisher's formula published by Dobson and Ikin 1946 and by Roberts 1948 .

Table XII.3 ABO system

Donors tested with anti-A_1, -A, -B and -A,B

Donors	Number tested		Phenotypes A_1	A_2	B	O	A_1B	A_2B
Indians*	666	No.	121	22	227	250	35	11
		%	(18,09)	(3,25)	(34,13)	(37,56)	(5,28)	(1,69)
Coloureds	541	No.	149	53	90	213	19	17
		%	(27,54)	(9,79)	(16,66)	(39,37)	(3,51)	(3,13)
Tamil	197	No.	34	9	71	73	7	3
		%	(17,26)	(4,57)	(36,04)	(37,06)	(3,55)	(1,52)
Telegu	101	No.	14	4	32	44	5	2
		%	(13,86)	(3,96)	(31,68)	(43,57)	(4,95)	(1,98)
Hindi	126	No.	27	2	45	45	6	1
		%	(21,42)	(1,59)	(35,72)	(35,72)	(4,76)	(0,79)
Moslem	242	No.	48	7	80	82	19	6
		%	(19,84)	(2,89)	(33,06)	(33,88)	(7,85)	(2,48)

* = Total Tamil + Telegu + Hindi + Moslem

Donors	Allele frequencies p_1	p_2	q	r	X^2 for ABs	p
Indians*	0,1247	0,0287	0,2327	0,6139	0,07	0,8
Coloureds	0,154	0,069	0,015	0,211	0,09	0,8
Tamil	0,1109	0,0351	0,2343	0,6197	1,06	0,5
Telegu	0,0985	0,0334	0,2155	0,6526	0,45	0,7
Hindi	0,1419	0,0141	0,2353	0,6087	0,57	0,5
Moslem	0,1488	0,0320	0,2462	0,5730	1,19	0,3

Allele frequencies calculated by the formulae given by Mourant, Kopec and Domaniewska-Sobczak, 1976, p51.

Table XII.4a ABO system

A_{bantu} in group A, and $A_{bantu}B$ in group AB, donors, estimated using anti-A_1 and by observing the agglutination pattern

	Number group A	A_{bantu}		Number group AB	$A_{bantu}B$	
Donors	tested	No.	%	tested	No.	%
Blacks	2499	110	(4,40)	409	62	(15,16)
Whites	1532	1	(0,06)	147	2	(1,36)
Coloureds	202	15	(2,77)	36	5	(0,92)

Table XII.4b ABO system

Subgroups of A in group A donors, estimated using anti-A_1 and by observing the agglutination pattern

		Phenotypes					
	Number	A_1		A_2		A_{bantu}	
Donors	tested	No.	%	No.	%	No.	%
Blacks	472	276	(58,47)	176	(37,29)	20	(4,24)
Whites	557	420	(75,40)	137	(24,60)	0	(0,00)

Table XII.5 ABO system

H antigen strength in group AB donors

		Phenotypes							
	Number	A_1B H-		A_1B H+		A_2B H-		A_2B H+	
Donors	tested	No.	%	No.	%	No.	%	No.	%
Blacks	102	33	(32,35)	19	(18,63)	21	(20,59)	29	(28,43)
Whites *	114	76	(66,67)	6	(5,26)	9	(7,89)	23	(20,18)
Indians *	112	33	(29,47)	57	(50,89)	4	(3,57)	18	(16,07)
Tamil	36	10	(ins)	19	(ins)	1	(ins)	6	(ins)
Telegu	20	6	(ins)	9	(ins)	2	(ins)	3	(ins)
Hindi	28	8	(ins)	17	(ins)	0	(ins)	3	(ins)
Moslem	28	9	(ins)	12	(ins)	1	(ins)	6	(ins)

ins = insufficient number

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.6 MNSs system

Donors tested with anti-M and anti-N only

Donors	Number tested	Phenotypes M No.	%	MN No.	%	N No.	%
Blacks	563	192	(34,10)	283	(50,27)	88	(15,63)
Whites	360	106	(29,44)	174	(48,33)	80	(22,22)
Indians*	514	193	(37,55)	260	(50,58)	61	(11,87)
Coloureds	528	155	(29,35)	270	(51,14)	103	(19,51)
Tamil	113	48	(42,48)	53	(46,90)	12	(10,62)
Telegu	109	37	(33,95)	58	(53,21)	14	(12,84)
Hindi	104	43	(41,35)	52	(50,00)	9	(8,65)
Moslem	188	65	(34,57)	97	(51,60)	26	(13,83)

* = Total Tamil + Telegu + Hindi + Moslem

Donors	Allele frequencies M	N	X^2 1df	p
Blacks	0,5924	0,4076	0,94	0,5
Whites	0,5361	0,4638	0,29	0,7
Indians*	0,6284	0,3716	3,55	0,1
Coloureds	0,5492	0,4508	0,57	0,5
Tamil	0,6593	0,3407	0,21	0,7
Telegu	0,6056	0,3944	1,45	0,2
Hindi	0,6635	0,3365	1,51	0,3
Moslem	0,6037	0,3963	1,15	0,3

Allele frequencies calculated according to the formulae of Race and Sanger, 1968, p56-57 and 89-90.

X^2 calculated for goodness of fit by the formula of Mourant, Kopec and Domaniewska-Sobczak, 1976, p48.

Table XII.7 MNSs system

Donors tested with anti-M, -N and -S only

Donors	Number tested		Phenotypes MS	Ms	MNS	MNs	NS	Ns
Blacks	563	No.	79	109	120	166	26	63
		%	(14,03)	(19,36)	(21,31)	(29,49)	(4,62)	(11,19)
Whites	277	No.	55	26	72	65	16	43
		%	(19,86)	(9,39)	(25,99)	(23,46)	(5,78)	(15,52)
Indians*	496	No.	126	67	135	113	24	31
		%	(25,40)	(13,51)	(27,22)	(22,78)	(4,84)	(6,25)
Tamil	105	No.	26	17	32	20	4	6
		%	(24,76)	(16,19)	(30,48)	(19,05)	(3,81)	(5,71)
Telegu	101	No.	28	15	21	28	5	4
		%	(27,73)	(14,85)	(20,79)	(27,72)	(4,95)	(3,96)
Hindi	100	No.	24	12	26	24	9	5
		%	(24,00)	(12,00)	(26,00)	(24,00)	(9,00)	(5,00)
Moslem	190	No.	48	23	56	41	6	16
		%	(25,26)	(12,11)	(29,47)	(21,58)	(3,16)	(8,42)

* = Total Tamil + Telegu + Hindi + Moslem

Donors	Allele frequencies MS	Ms	NS	Ns	X^2 (2df)	p
Blacks	0,1512	0,4367	0,0739	0,3382	3,4	0,2
Whites	0,2353	0,3044	0,0692	0,3911	0,04	0,98
Indians*	0,2598	0,3793	0,0880	0,2729	3,6	0,2
Tamil	0,2676	0,3896	0,0925	0,2503	2,6	0,3
Telegu	0,2312	0,4372	0,0867	0,2449	3,6	0,2
Hindi	0,2247	0,3853	0,1350	0,2550	2,0	0,5
Moslem	0,2844	0,3445	0,0667	0,3044	2,2	0,5

Allele frequencies calculated according to the formula of Mourant, Kopec and Domaniewska-Sobczak, 1976, p52-53.

X^2 calculated for goodness of fit by the formula of Mourant, Kopec and Domaniewska-Sobczak, 1976, p48.

Table XII.8 MNSs system Donors tested with anti-M, anti-N, anti-S and anti-s

Donors	Number tested		Phenotypes MS	MSs	Ms	MNS	MNSs	MNs	NS	NSs	Ns
Indians*	618	No.	42	106	88	28	125	141	8	30	50
		%	(6,80)	(17,15)	(14,24)	(4,53)	(20,23)	(22,82)	(1,29)	(4,85)	(8,09)
Coloureds	528	No.	11	58	86	32	91	147	5	28	70
		%	(2,08)	(10,98)	(16,29)	(6,06)	(17,24)	(27,84)	(0,95)	(5,30)	(13,26)
Tamil	199	No.	11	33	29	8	40	48	3	12	15
		%	(5,53)	(16,58)	(14,57)	(4,02)	(20,10)	(24,12)	(1,51)	(6,03)	(7,54)
Telegu	102	No.	10	19	12	5	19	23	1	4	9
		%	(9,80)	(18,63)	(11,77)	(4,90)	(18,63)	(22,55)	(0,98)	(3,92)	(8,82)
Hindi	113	No.	8	19	20	3	19	29	1	7	7
		%	(7,08)	(16,81)	(17,71)	(2,66)	(16,81)	(25,66)	(0,89)	(6,19)	(6,19)
Moslem	204	No.	13	35	27	12	47	41	3	7	19
		%	(6,37)	(17,16)	(13,24)	(5,88)	(23,04)	(20,10)	(1,47)	(3,43)	(9,31)

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.8 continued MNSs system

Donors	Allele frequencies MS	Ms	NS	Ns	X^2 (5df)	p
Indians*	0,2421	0,3777	0,0952	0,2850	3,3	0,7
Coloureds	0,1465	0,4027	0,1120	0,3388	19,2	0,01
.....					.....	
Tamil	0,2206	0,3874	0,1035	0,2885	1,1	0,95
Telegu	0,2857	0,3467	0,0770	0,2906	1,6	0,9
Hindi	0,2169	0,4248	0,0884	0,2699	3,2	0,7
Moslem	0,2553	0,3575	0,1000	0,2872	3,3	0,7

Allele frequencies calculated according to the formulae of Mourant, Kopec and Domaniewska-Sobczak, 1976, p53.

Table XII.9 MNSs system

Donors tested with anti-M_1

Donors	Phenotype: Number tested	M_1 No.	M_1 %	Allele frequencies: M_1	non-M_1
Indians*	579	10	(1,73)	0,0087	0,9913
.....	.....	.....	.....	.....	.....
Tamil	215	1	(0,47)	0,0024	0,9976
Telegu	105	2	(1,90)	0,0095	0,9905
Hindi	155	2	(1,29)	0,0065	0,9935
Moslem	104	5	(4,81)	0,0243	0,9757

* = Total Tamil + Telegu + Hindi + Moslem

Allele frequencies calculated according to the formula for two alleles and two phenotypes in Mourant, Kopec and Domaniewska-Sobczak, 1976, p49.

Table XII.10 MNSs system

Donors tested with anti-He (Henshaw)

Donors	Phenotype: Number tested	He+ No.	He+ %	Allele frequencies: He+	He-
Blacks	1121	45	(4,01)	0,0203	0,9797

Distribution of He+ in MNSs

Donors	Number tested	Phenotypes: MS	MSs	Ms	MNS	MNSs	MNs	NS	NSs	Ns
He+ Blacks	45	4	18	0	6	15	0	0	2	0

Table XII.11 P system

Donors tested with anti-P_1

Donors	Number tested	Phenotypes P_1 No.	%	P_2 No.	%	Allele frequencies P_1	P_2
Blacks	1027	969	(94,35)	58	(5,65)	0,7623	0,2377
Whites	426	335	(78,64)	91	(21,36)	0,5378	0,4622
Indians*	716	523	(73,04)	193	(26,96)	0,4808	0,5192
.....						.....	
Tamil	211	153	(72,51)	58	(27,49)	0,4757	0,5243
Telegu	115	80	(69,57)	35	(30,43)	0,4484	0,5516
Hindi	142	117	(82,39)	25	(17,61)	0,5804	0,4196
Moslem	248	173	(69,76)	75	(30,24)	0,4501	0,5499

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.12 Rh system

Male donors tested with anti-D: D-negatives tested with anti-CD and anti-E and for D^u

Donors:	Blacks		Whites		Indians*		Tamil		Telegu		Hindi		Moslem	
Number tested:	6057		1884		2442		987		398		570		487	
Phenotypes	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
(95,29) 939 (95,14)	370	(92,96)	553	(97,02)	465	(95,48)								
D^uCe (low grade)	14	(0,23)	0	(0,00)	6	(0,24)	2	(0,20)	4	(1.01)	0	(0,00)	0	(0,00)
D^uce (low grade)	26	(0,43)	0	(0,00)	0	(0,00)								
D^ucE (low grade)	0	(0,00)	7	(0,37)	0	(0,00)								
dCe	50	(0,83)	12	(0,64)	7	(0,29)	1	(0,10)	3	(0,75)	2	(0,35)	1	(0,20)
dcE	0	(0,00)	6	(0,32)	1	(0,04)	0	(0,00)	0	(0,00)	0	(0,00)	1	(0,20)
dce	152	(2,51)	274	(14,54)	101	(4,14)	45	(4,56)	21	(5,28)	15	(2,63)	20	(4,11)

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.13 Rh system Donors tested with anti-D, -C, -E, -c and -e. D-negatives were tested for D^u

Donors:	Blacks		Whites		Indians*		Coloureds		Tamil		Telegu		Hindi		Moslem	
Number tested:	530		334		1168		541		423		171		260		314	
Phenotypes	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
DCe/dce or DCe/Dce	71	(13,40)	120	(35,93)	389	(33,30)	200	(36,97)	138	(32,62)	66	(38,60)	78	(30,00)	107	(34,07)
D^uCe/dce (low grade)	1	(0,19)	0	(0,00)	0	(0,00)	7	(1,29)								
DCe/DCe	8	(1,51)	57	(17,07)	523	(44,78)	65	(12,01)	201	(47,52)	70	(40,94)	138	(53,08)	114	(36,30)
DcE/dce or DcE/Dce	82	(15,47)	38	(11,38)	50	(4,28)	66	(12,20)	13	(3,07)	8	(3,08)	8	(3,08)	21	(6,69)
D^ucE/dce (low grade)	0	(0,00)	1	(0,30)	0	(0,00)	2	(0,37)								
DcE/DcE	8	(1,51)	17	(5,09)	16	(1,37)	8	(1,48)	4	(0,94)	4	(2,34)	1	(0,38)	7	(2,23)
dce/dce	13	(2,45)	49	(14,67)	49	(4,20)	28	(5,18)	15	(3,55)	5	(2,92)	14	(5,38)	15	(4,78)
DCe/DcE	15	(2,83)	42	(12,57)	111	(9,50)	35	(6,47)	44	(10,40)	14	(8,19)	14	(5,38)	39	(12,42)
Dce/dce or Dce/Dce	325	(61,32)	7	(2,09)	18	(1,54)	113	(20,89)	4	(0,95)	3	(1,75)	4	(1,54)	7	(2,23)
D^uce/dce or D^uce/D^uce (low grade)	2	(0,38)	0	(0,00)	0	(0,00)	6	(1,11)								
dCe/dce	5	(0,94)	2	(0,60)	7	(0,60)	8	(1,48)	3	(0,71)	0	(0,00)	2	(0,77)	2	(0,64)
dcE/dce	0	(0,00)	1	(0,30)	1	(0,09)	0	(0,00)	0	(0,00)	0	(0,00)	0	(0,00)	1	(0,32)
DCE/DCe	0	(0,00)	0	(0,00)	4	(0,34)	3	(0,55)	1	(0,24)	1	(0,58)	1	(0,38)	1	(0,32)
X^2	5,18		7,39		15,03		0,47		1,02		2,38		3,55		1,27	
	0,5 (5df)		0,3 (6df)		0,05 (7df)		0,99 (5df)		0,98 5df		0,5 3df		0,7 5df		0,9 5df	

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.13 continued Rh system

Donors tested with anti-D, -C, -E, -c and -e. All D-negatives tested for D^u

Haplotype frequencies (D^u included with D+)

Haplotypes	Blacks	Whites	Indians*	Coloureds	Tamil	Telegu	Hindi	Moslem
DCe	0,0777	0,4083	0,6511	0,3258	0,6755	0,6446	0,6966	0,5847
DcE	0,1066	0,1706	0,0803	0,1067	0,0762	0,0861	0,0454	0,1126
dce	0,1547	0,3837	0,2138	0,2302	0,2021	0,2093	0,2109	0,2310
Dce	0,6368	0,0264	0,0361	0,2971	0,0254	0,0554	0,0283	0,0487
dCe	0,0242	0,0079	0,0145	0,0325	0,0190	0,0000	0,0162	0,0146
dcE	0,0000	0,0030	0,0015	0,0000	0,0000	0,0000	0,0000	0,0058
dCE+DCE	0,0000	0,0000	0,0029	0,0077	0,0018	0,0046	0,0026	0,0026

Haplotype frequencies calculated according to the formulae of Mourant, Kopec and Domaniewska-Sobczak, 1976, p53-55.

Table XII.14 Rh system

D+ donors tested with anti-C^W

Donors	Phenotype Number tested	C^W+ No.	%	Distribution of C^W+ DCe/DCe	DCe/dce	DCe/DcE	Other
Blacks	46	0	(ins)	0	0	0	0
Whites	154	5	(3,25)	1	3	1	0
Indians*	1556	8	(0,51)	5	2	1	0
.....				.....			
Tamil	627	0	(0,00)	0	0	0	0
Telegu	264	0	(0,00)	0	0	0	0
Hindi	344	2	(0,58)	2	0	0	0
Moslem	321	6	(1,87)	3	2	1	0

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.15 Rh system

Donors with e+ red cells tested with anti-hr^S

Donors	Phenotype Number tested	e+ e+,hr^S- No.	%	Distribution of hr^S- DcE/ce	Dce/ce
Blacks	573	9	(1,57)	4	5
Whites	95	0	(ins)	0	0
Indians*	1446	0	(0,00)	0	0

Table XII.16 Lutheran system

Donors tested with anti-Lu^a

Donors	Phenotype: Number tested	Lu(a+) No.	Lu(a+) %	Allele frequencies: Lu^a	Lu^b
Blacks	540	36	(6,67)	0,0339	0,9661
Whites	423	28	(6,62)	0,0337	0,9663
Indians	725	0	(0,00)	0,0000	1,0000

Table XII.17 Kell system

Donors tested with anti-K

Donors	Phenotype: Number tested	K+ No.	K+ %	Allele frequencies: K	$k+K^o$
Blacks	501	11	(2,20)	0,0111	0,9889
Whites *	568	48	(8,45)	0,0432	0,9568
Indians *	1165	28	(2,40)	0,0121	0,9879
Coloureds	541	21	(3,88)	0,0194	0,9806
.....	.....	.....	.....	.....	.....
Tamil	399	8	(2,00)	0,0101	0,9899
Telegu	181	2	(1,10)	0,0055	0,9945
Hindi	289	2	(0,69)	0,0035	0,9965
Moslem	296	16	(5,40)	0,0274	0,9726

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.18 Kell system

Donors tested with anti-Kp^a

Donors	Phenotype: Number tested	Kp(a+) No.	Kp(a+) %	Allele frequencies: Kp^a	Allele frequencies: Kp^b+K^o
Blacks	500	0	(0,00)	0,0000	1,0000
Whites	335	8	(2,39)	0,0120	0,9880
Indians *	1041	3	(0,29)	0,0015	0,9985
Coloureds	539	2	(0,37)	0,0018	0,9982
.....	.....	.....	.....	.....	.....
Tamil	358	0	(0,00)	0,0000	1,0000
Telegu	148	0	(0,00)	0,0000	1,0000
Hindi	254	3	(1,18)	0,0059	0,9941
Moslem	281	0	(0,00)	0,0000	1,0000

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.19 Lewis and Secretor systems

Secretion of H substance by donors of all ABO groups whose saliva was tested with anti-H by inhibition technique

Donors	Number tested	Phenotypes: H substance secreted No.	%	H substance not secreted No.	%	Allele frequencies: Se	se
Blacks+	171	125	(73,10)	46	(26,90)	0,4814	0,5186
Blacks	163	122	(74,85)	41	(25,15)	0,4985	0,5015
Whites+	74	56	(ins)	18	(ins)	(insufficient)	

ins = insufficient

\+ = Paper 14 by Moores and Brain, 1968

Table XII.20 Lewis and Secretor systems

Secretion of Le^a substance by donors of all ABO groups whose saliva was tested with anti-Le^a by inhibition technique

Donors	Number tested	Le^a substance detected No.	%	Le^a substance not detected No.	%
Blacks+	171	128	(74,85)	43	(25,15)
Blacks	163	127	(77,91)	36	(22,09)

\+ = Paper 14 by Moores and Brain, 1968

Table XII.21 Lewis and Secretor systems

Donors of all ABO groups and known ABH and Lewis secretor phenotypes whose red cells were tested with anti-Le^a, anti-Le^{bL} and anti-Le^X. No variation in Lewis group distribution within particular ABO phenotypes was noted

Donors	Number tested	Substances secreted			Red cell phenotypes					
		ABH	Le^a	Le^b	Le(a-b+) No.	%	Le(a-b-) No.	%	Le(a+b-) No.	%
Blacks+	171	+	+	+	83	(48.54)	7	(4,09)	0	(0,00)
		+	-	+	10	(5,85)	25	(14,62)	0	(0,00)
		-	+	-	0	(0,00)	0	(0,00)	38	(22,22)
		-	-	-	0	(0,00)	8	(4,68)	0	(0,00)
Blacks	163	+	+	+	86	(52,76)	0	(0,00)	0	(0,00)
		+	+	-	0	(0,00)	2	(1,23)	0	(0,00)*
		+	-	+	0	(0,00)	35	(21,47)	0	(0,00)
		-	+	-	0	(0,00)	8	(4,91)*	31	(19,02)
		-	-	-	0	(0,00)	1	(0,61)	0	(0,00)
Whites+	74	+			51	(68,92)	5	(6,76)		
		-					1	(1,35)	17	(22,97)
Total:					86	(52,76)	46	(28,22)	31	(19,02)

* = unexpected result + = Paper 14 by Moores and Brain, 1968

Table XII.22

Lewis and Secretor systems

Group O donors' red cells

tested with anti-Le^a only

Donors	Phenotype Number tested	Le(a+) No.	%
Indians*	427	103	(24,12)
Tamil	125	33	(26,40)
Telegu	102	25	(24,51)
Hindi	100	22	(22,00)
Moslem	100	23	(23,00)

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.23 Lewis and Secretor systems

Donors' red cells tested with anti-Le^a, anti-Le^{bL} and anti-Le^X

Donors	ABO group	Number tested	Phenotypes Le(a-b+) No.	%	Le(a+b-) No.	%	Le(a-b-) No.	%	Le(a+b+) No.	%
Blacks+	all	171	93	(54,39)	38	(22,22)	40	(23,39)	0	(0,00)
Blacks	all	163	81	(49,69)	31	(19,02)	46	(28,22)	5	(3,07)
Whites+*	all	74	51	(ins)	17	(ins)	6	(ins)	0	(ins)
Indians	0	1175	719	(61,19)	281	(23,91)	169	(14,38)	6	(0,51)
Tamil	0	426	228	(53,52)	125	(29,34)	69	(16,20)	4	(0,94)
Telegu	0	198	125	(63,13)	43	(21,72)	28	(14,14)	2	(1,01)
Hindi	0	269	167	(62,08)	63	(23,42)	39	(14,50)	0	(0,00)
Moslem	0	282	199	(70,57)	50	(17,73)	33	(11,70)	0	(0,00)

\+ = Paper 14 by Moores and Brain, 1968

* = Total Tamil + Telegu + Hindi + Moslem

ins = insufficient

Donors	Allele frequencies Le	le	Donors	Le	le
Blacks+	0,5154	0,4836	Tamil	0,5975	0,4025
Blacks	0,4688	0,5312	Telegu	0,6240	0,3760
Whites+*	(insufficient)		Hindi	0,6192	0,3808
Indians	0,6208	0,3792	Moslem	0,6580	0,3420

Frequencies calculated according to Mourant, Kopec and Domanieskwa-Sobczak, 1976, p 56, despite the presence of Le(a+b+) which suggested that the current theory of Le/le gene inheritance was incomplete.

Table XII.24 Duffy system

Donors tested with anti-Fy^a

Donors	Phenotype: Number tested	Fy(a+) No.	Fy(a+) %	Allele frequencies: Fy^a	Allele frequencies: Fy^b+Fy
Blacks	598	87	(14,55)	0,0756	0,9244
Whites	357	237	(66,39)	0,4203	0,5797
Indians*	304	264	(86,84)	0,6372	0,3628
Indians	509	434	(85,26)	0,6161	0,3839
Coloureds	539	295	(54,73)	0,3272	0,6728
.....	.....	.....	.....	.....	.....
Tamil	144	130	(90,28)	0,6882	0,3118
Telegu	118	112	(94,91)	0,7744	0,2256
Hindi	147	130	(88,44)	0,6600	0,3400
Moslem	100	72	(72,00)	0,4709	0,5291

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.25 Duffy system

Donors tested with anti-Fy^a, anti-Fy^b and anti-Fy3

Phenotypes

Donors	Number tested	Fy(a+b-) No.	%	Fy(a-b+) No.	%	Fy(a+b+) No.	%	Fy(a-b-) No.	%
Blacks	273	26	(9,52)	64	(23,44)	6	(2,20)	177	(64,84)
Whites *	36	6	(ins)	16	(ins)	14	(ins)	0	(ins)
Indians *	391	170	(43,48)	55	(14,07)	165	(42,20)	1	(0,25)
Tamil	114	47	(41,23)	15	(13,16)	52	(45,61)	0	(0,00)
Telegu	76	42	(ins)	9	(ins)	25	(ins)	0	(ins)
Hindi	97	40	(ins)	9	(ins)	48	(ins)	0	(ins)
Moslem	104	41	(39,42)	22	(21,16)	40	(38,46)	1	(0,96)

* = Total Tamil + Telegu + Hindi + Moslem

ins = insufficient

Allele frequencies

Donors	Fy^a	Fy^b	Fy
Blacks	0,0603	0,1375	0,8022
Whites *	(insufficient)		
Indians *	0,6184	0,3370	0,0446
Tamil	0,6388	0,3588	0,0024
Telegu	(insufficient)		
Hindi	(insufficient)		
Moslem	0,5318	0,3659	0,1023

Table XII.26 Kidd system

Donors tested with anti-Jk^b

Donors	Phenotype: Number tested	Phenotype: Jk(b+) No.	Phenotype: Jk(b+) %	Allele frequencies: Jk^a+Jk	Allele frequencies: Jk^b
Indians*	535	301	(56,26)	0,6614	0,3386
Tamil	124	69	(55,64)	0,6660	0,3340
Telegu	108	58	(53,70)	0,6804	0,3196
Hindi	125	69	(55,20)	0,6693	0,3307
Moslem	178	105	(58,99)	0,6404	0,3596

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.27 Xg^a antigen

Donors tested with anti-Xg^a

Phenotypes in males

Donors	Number tested	Xg(a+) No.	%	Xg(a-) No.	%
Blacks *	110	48	(43,64)	62	(56,36)
Indians *	412	294	(71,36)	118	(28,64)
.....					
Tamil	105	81	(77,14)	24	(22,86)
Telegu	106	77	(72,64)	29	(27,36)
Hindi	101	72	(71,29)	29	(28,71)
Moslem	100	64	(64,00)	36	(36,00)

* = Total Tamil + Telegu + Hindi + Moslem

Donors tested with anti-Xg^a

Phenotypes in females

Donors	Number tested	Xg(a+) No.	%	Xg(a-) No.	%
Blacks	40	31	(ins)	9	(ins)

ins = insufficient

Allele frequencies in males

Donors	Xg^a	Xg
Blacks *	0,4364	0,5636
Indians *	0,7136	0,2864
.....		
Tamil	0,7714	0,2286
Telegu	0,7264	0,2736
Hindi	0,7129	0,2871
Moslem	0,6400	0,3600

Table XII.28 Colton system

Donors tested with anti-Co^b; all Co(b+) were tested with anti-Co^a

Donors	Number tested	Phenotypes Co(a+b+) No.	%	Co(a-) No.	%	Allele frequencies Co^a + Co	Co^b
Indians*	789	9	(1,14)	780	(98,86)	0,0057	0,9943
Tamil	275	5	(1,82)	270	(98,18)	0,0092	0,9908
Telegu	144	1	(0,69)	143	(99,31)	0,0035	0,9965
Hindi	154	1	(0,65)	153	(99,35)	0,0033	0,9967
Moslem	216	2	(0,93)	214	(99,07)	0,0047	0,9953

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.29 Diego system

Donors tested with anti-Di^a

Donors	Number tested	Phenotype Di(a+) No.	%
Indians*	129	2	(1,55)
Tamil	58	1	(ins)
Telegu	28	0	(ins)
Hindi	30	1	(ins)
Moslem	13	0	(ins)

* = Total Tamil + Telegu + Hindi + Moslem

ins = insufficient

Table XII.30 In system

Donors tested with anti-In[a]; all In(a+) were tested with anti-In[b]

Donors	Number tested	Phenotypes In(a+b+) No.	%	In(a-) No.	%	Allele frequencies In[a]	In
Indians*	583	13	(2,23)	570	(97,77)	0,0112	0,9888
.....						.....	.....
Tamil	228	4	(1,75)	224	(98,25)	0,0088	0,9912
Telegu	103	3	(2,91)	100	(97,09)	0,0147	0,9853
Hindi	191	5	(2,62)	186	(97,38)	0,0132	0,9868
Moslem	61	1	(ins)	60	(ins)	(insufficient)	

* = Total Tamil + Telegu + Hindi + Moslem

ins = insufficient

Table XII.31 Sd[a] (Sid) groups

Donors tested with potent anti-Sd[a]

Donors	Number tested	Phenotypes Sd(a+) No.	%	Sd(a-) No.	%
Blacks	300	254	(84,67)	46	(15,33)
Whites	114	109	(95,61)	5	(4,39)
Indians*	577	499	(86,48)	78	(13,52)
.....					
Tamil	201	178	(88,56)	23	(11,44)
Telegu	107	92	(85,98)	15	(14,02)
Hindi	135	111	(82,22)	24	(17,78)
Moslem	134	118	(88,06)	16	(11,94)

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.32 Sd^a (Sid) groups

Donors tested with anti-A_1 Dolichos biflorus lectin to detect Sd(a+++) red cells

Donors	Phenotype: Number tested	Sd(a+++) No.	Sd(a+++) %
Indians*	571	11	(1,93)
Tamil	185	4	(2,16)
Telegu	100	3	(3,00)
Hindi	122	3	(2,46)
Moslem	164	1	(0,61)

* = Total Tamil + Telegu + Hindi + Moslem

Table XII.33 I antigen

Donors tested with anti-I

Donors	Phenotypes: Number tested	I+ No.	I+ %	I- No.	I- %
Blacks	479	476	(99,37)	3	(0,63)
Whites	557	557	(100,00)	0	(0,00)

CHAPTER XIII

THE SOUTHERN AFRICAN ODYSSEY

Surely no land is more richly-endowed with interesting and unusual blood groups than southern Africa! Among its many populations, each has its own complement of rare genes and phenotypes; and the author was privileged to have worked in Natal as a blood group serologist for the past 30 years. In the ABO system, the A subtypes A_x and A_y, and the B subtypes B_m, B_m-like and B_3-like, were "collectors' pieces". The A subtype, A_{bantu}, was brilliantly shown by Brain [1966] to be a unique phenotype. Arguments still take place about whether or not A_{bantu} should preferably have been named $A_{Khoisan}$ [Jenkins, 1972]. Oriol, Samuelsson and Messeter [1990] have since shown that the few A epitopes on A_{bantu} red cells are probably all A type 1.

The discovery that the "Bombay" O_h phenotype existed in South Africa had serious implications for the blood banks there. As the plasma contained anti-A, anti-B and anti-H, only the immediate relatives of the propositi and others with the same phenotype could provide compatible blood. As each O_h appeared, the author made a detailed family study. Some members with O_h red cells who were found in this way were young children, but one day they would be old enough to donate blood. The Rh negative O_h and Le(a-b-) O_h were further "collectors' pieces", for at the time, only one other family with both O_h Le(a+b-) and O_h Le(a-b-) siblings was known world-wide. The total absence of A, B and H antigens from the red cells of the South African O_h Indians identified them as the "Classical" type. When the South African Rare Donor File was founded, they were among the first donors included. The File was useful when donations of rare blood were required urgently. The Natal Blood Transfusion Service supplies donations of rare blood to blood banks not only in the Republic but throughout the world.

Seven of the 24 O_h Indians identified in Natal donated samples of their blood and saliva for research overseas. The research led, among other features, to the identification of the

biochemical background of the ABO and Lewis blood groups [Schenkel-Brunner, Chester and Watkins, 1972; Race and Watkins, 1972a and b; Rodier, Lopez, Liberge et al., 1974; Chester, Yates and Watkins, 1976; Dodd and Lincoln, 1978; Watkins, 1980]. Until the studies of Gerard, Vitrac, Le Pendu et al. in 1982 and those of Le Pendu, Gerard, Vitrac et al. and of Le Pendu, Clamagirand-Mulet, Cartron et al. in 1983 in the diverse populations of Réunion Island, the Indians in Natal comprised the largest collection of O_h beyond the borders of India [Oriol, 1990]. The data from Natal was included by Oriol and his colleagues [1990] in their studies which showed, contrary to the then current belief, that the "Classical" O_h phenotype was associated with *se/se* and not with suppressed *Se/Se* or *Se/se* genotypes in the Secretor system. Le Pendu et al.[1983] also described the new "Réunion" O_h phenotype, in which the plasma contained a small quantity of H enzyme. In the "Classical" O_h phenotype, the plasma contained none.

The three Indians with para-Bombay red cells, type O^{A}_{Hm}, O^{B}_{Hm} and O^{o}_{Hm} respectively, were identified in Natal as the result of the anti-H in their plasma. In two, this antibody made it difficult to find compatible blood for them. No records of other examples of these phenotypes in southern Africa have been found.

Blood donors with S-s-U- red cells were soon added to the South African Rare Donor File. The number of Blacks with this phenotype was lower in Natal than in the other provinces and other countries to the north. The Natal U- Indians made up for this by being loyal donors for years. The Indian and Black family studies proved that U- red cells were due to the inheritance of a *u* (or S^{u}) and not to an inhibitor gene. The predominance of *Nu* over *Mu* genes was interesting, for *MU* usually outnumbered *NU* genes. In disputed paternity cases, when *u* was suspected, carefully measured and controlled titrations with anti-S and anti-s were needed to detect *S/s* gene dosage. The source, age and condition of the red cells had also to be as exactly comparable as possible. These studies were brought to a close before the author's small stock

of anti-s became too depleted.

The discovery that the gene complex *Dantu* was present in southern Africa opened up another fascinating field of investigation. Mr Dantu, in whom the phenotype was first identified, lived in Canada. A few Negroes in the United States had also been grouped as Dantu+ [Contreras et al. 1984]. During the author's phenotype and gene frequency studies in Natal Coloured blood donors, the red cells of one donor gave unusual MNSs results. In the follow-up studies, by chance, a sample of anti-Dantu was included. When the donor's red cells gave a strong positive result, an excited exchange of letters with Canada ensued. Mr Dantu was a person of mixed ethnic origin, and he had originally lived at the Cape! The author studied the inheritance of *Dantu* in the Natal Coloured donor's large family and made many comparative antigen dosage titrations. With the welcome advice and support of John Moulds (Gamma Biologicals, Houston, Texas, USA), the unusual serological results were soon understood. Further individuals with Dantu+ red cells have since been found, both in Natal and at the Cape, and a paper about them is in the process of being written. Meanwhile, the search for the population origin of *Dantu* continues. As Dantu+ red cells have been identified at the Cape but none yet in other southern African populations, it is possible, but not yet proved, that the complex arose as a mutation (or mutations) in the Khoi people. The Khoi are an ancient southern African race, whose ancestors were present at the Cape in the early years of White settlement there. The genes of the Khoi are present in the modern Cape Coloured population [Nurse, Weiner and Jenkins, 1985, p221-224].

The woman with Dantu+ Gerbich- red cells was a further "first" for southern Africa. As the genes encoding Dantu and Gerbich are carried on different chromosomes, their combined presence in a single individual, although an exceedingly rare event, had occurred by chance.

Colleagues in other countries, as well as the author, searched in vain in their populations for an anti-Tj^a-like haemolysin

similar to the one found in Perth. The haemolysin may be associated in some way with a parasite of Western Australian sheep. The antigen P_1H had apparently not been recorded elsewhere. As three examples of the corresponding P_1H antibody were identified in Natal, this specificity may be relatively common there. The P and ABO antigens are closely associated at the molecular level [Watkins, 1980, p109], and an antibody for a specific interaction product was possible. The inheritance of P_1H was traced in three families: further work is clearly indicated.

The discovery in Natal of the third example of anti-hr^s in Mrs Sakwe's serum re-stimulated the interest which Dr Shapiro's original example [1960] of this antibody had aroused in the author in the variants of Rh antigen e. Fortunately, Mrs Sakwe's case was mild, and Dr Shapiro had reported almost no "Hr-like" antibodies in her serum. Whether the weakness of the "Hr-like" antibody was due to Mrs Sakwe's R_2r phenotype or not (R^2 encodes Rh18 weakly) is a matter still being considered. The work with Mrs Sakwe's blood led to the founding of the Blood Group Reference Laboratory at the Natal Blood Transfusion Service. After Dr Shapiro described anti-hr^B in 1972, the author collected and studied many sera which she believed also had this specificity. Their anti-Rh34 specificity instead, became clear when red cells with ordinary Rh phenotypes were found to be Rh:-34. Like other Rh antibodies and their corresponding antigens, anti-Rh34 and Rh:34 were specific entities; no evidence was found that anti-Rh34 was a mixture of two antibodies, anti-hr^B and "Hr-like" or that only D--, Dc- (Rh "deleted") and Rh_{null} red cells were compatible [Shapiro, 1972]. The world-wide confusion about "anti-hr^B" should now have been resolved. The low frequency antigen, STEM, which is linked to Rh and closely associated with the phenotypes hr^s- and Rh:-34, unexpectedly subdivided both phenotypes into two kinds: STEM+ and STEM-. When patients have anti-hr^s or anti-Rh34 in their plasma, STEM is a useful marker for identifying compatible blood.

The discovery that the Rh *D*-- haplotype was present at the Cape proved the value of having persisted with the disputed paternity investigations in which unusual exclusions had been identified in the Rh system. A *D*-- homozygote should soon be found there. *D*-- readily revealed when an $\hat{R}^{o}$ or R^{-34} haplotype had been inherited *in trans*: these genotypes were the first of their kind recorded. The Rh *Dc*- haplotype had also not been reported before from southern Africa, but it was some years before the unusual antigens produced by the heterozygotes were fathomed. The first South African example of Rh_{null} red cells gave the author a splendid opportunity to use her entire collection of Rh grouping reagents.

Oval-shaped red cells, more commonly known as elliptocytes, caused much excitement when they were unexpectedly identified in a donor's blood. The relevant gene or genes were seen to be linked to a *r* (*dce*) haplotype. The condition is evidently rare in southern Africa for no others have been brought to the author's attention.

The rare haplotype, K^{o}, was revealed in the heterozygous state when anti-Kp^{b} was unexpectedly detected in addition to anti-CD in a White blood donor's plasma. Very few people with homozygous K^{o} genes are known. In the family study, through the kindness of Derek Ford (Canberra, Australia) the author was able to use the curious anti-k reagent, Rossner, which gives weaker than usual results with the red cells of K^{o} heterozygotes. The discovery that the rare Js(b-) phenotype had a 1% frequency in Zimbabwian Blacks was useful when compatible blood was needed for patients whose plasma contained anti-Js^{b}.

One of the only two examples of Le(a-b-c-d-) red cells so far reported was discovered in a sibling of the Black dispermic chimera, Durban III, during family ABO serum transferase studies in London. In other studies, rare Wd(a+) red cells were surprisingly identified in the Hei//om people of Namibia. The Wd^{a} gene had been known previously only in the Hutterites of Canada and a Dutch family in Holland. Many Zulus in Natal

were found to have McC(e+) red cells and, in the first South African case of CDA type II, the patient's weak I and markedly enhanced i antigens helped confirm the identity of her haematological disorder.

Southern Africa is noteworthy for having produced three XX/XX dispermic chimeras, each with two populations of cells in the blood. The chimeras are three of the only four with normal female karyotypes known. The fourth, discovered elsewhere, had only one population of cells in her blood. The first southern African chimera was identified in Durban when an incomplete anti-D reagent gave a weak positive result in an Rh phenotyping test. The second was found through curiosity during karyotyping studies for suspected microcephaly in a family member, and the third when her blood donation was grouped as O but no evidence of anti-A,B was detected in her plasma. Although the exceptional patchy skin pigmentation in Durban I and Durban III was reported both in a dermatological journal and at a dermatological congress, no more examples have been brought to the author's attention.

Polyagglutinable red cells due to T-sensitization were identified in a Black woman in Durban, who astonishingly also had anti-T in her serum. Polyagglutinable red cells of a new type were also discovered in association with very rare haemoglobin M - type Hyde Park in a family in Pietermaritzburg. The latter case led to many biological and other studies being made in Britain with the blood of a family member. Pending the identification of polyagglutinable red cells of a similar type in other persons with the same haemoglobin, the family in Pietermaritzburg is unique.

The blood group phenotypes in the Black, White, Indian and Coloured blood donors of Natal were studied with many reagents, and their phenotype and gene frequencies calculated. This information should prove useful not only to blood bank workers but others in different fields.

The southern African odyssey is by no means over yet. The author still has much to publish, and she is certain that many more blood groups and conditions wait to be found. It is only necessary to keep one's eyes open. In the South African Medical Journal in 1952 (volume 26, page 239), O.E. Budtz-Olsen remarked

"The origin and diversity of mankind must always remain a problem of enduring fascination...".

The author enthusiastically agrees!

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APPENDIX

Previous Studies in Populations of Natal and Zululand

ABO System

Population and author	Habitation	Number tested	% Phenotype Frequency				Allele Frequency			AB subjects (calculated to nearest whole number		X^2	P
			A	B	O	AB	p	q	r	Observed	Expected		
Negroes (Zulus) Elsdon-Dew, 1936	Northern Zululand	500	24,6	21,6	51,8	2,0	0,1441	0,1266	0,7293	10	21	4,3	0,05
Negroes (Zulus) Zoutendyk, 1955	Northern Natal	322	33,5	23,3	37,6	5,6	0,2209	0,1575	0,6216	18	25	1,2	0,3
Negroes (African women) Hirsch, 1958	Natal	2 526	28,3	20,8	46,3	4,6	0,1811	0,1363	0,6826	116	128	0,84	0,5
Negroes Natal Blood Transfusion Service, 1960	Natal	3 580	31,6	19,2	44,3	4,8	0,2028	0,1283	0,6659	172	193	1,68	0,2
Negroes (Bantu women) Buckwalter et al., 1961	Natal	7 105	28,4	21,5	45,0	5,0	0,1847	0,1430	0,6723	359	382	1,03	0,5
Negroes (Bantu) Brain, 1966	Durban	867	30,7	19,4	43,8	6,1	0,2050	0,1369	0,6618	53	46	0,61	0,5

APPENDIX, continued

Previous studies in Populations of Natal and Zululand

Rh System

Donors tested with anti-CD, -D, -E and for D^u

Population	Habitation	Number tested	D+ No.	D+ %	Low grade D^u No.	Low grade D^u %	Cde No.	Cde %	cdE No.	cdE %	cde No.	cde %
Negro females Hirsch, 1958	Durban	2 526	2 407	95,30	3	0,10	D-: No.: 116	%: 4,60				
Negro donors, (males and females) Natal Blood Transfusion Service, 1960	Natal	3 583	3 371	94,06	82	2,29	D-: No.: 130	%: 3,63				

Donors tested with anti-C, -D, -E, -c, -e and for D^u (D^u included with D+)

Population	Habitation	Number tested	CDe/cde or CDe/cDe	CDe/CDe	cDE/cde or cDE/cDe	cDE/cDE	cde/cde	CDe/cDE	cDe/cde or cDe/cDe	Cde/cde	cdE/cde	Cde/cdE
Zulus Shapiro, 1951	Johannesburg	403	13,65	0,00	11,16	0,00	3,97	5,46	63,52	2,23	0,00	0,00