PERTUSSIS VACCINATION OF AFRICAN INFANTS USING ACELLULAR AND WHOLE-CELL VACCINES

by

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SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF MEDICINE

UNIVERSITY OF NATAL, DURBAN
1991

In this research the statistical planning and analyses, and recommendations arising from these analyses, have been done in consultation with the Institute for Biostatistics of the Medical Research Council.

DECLARATION

This thesis is the candidates own original work and has not been presented for any degree at another university.

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DATE: 20 th December 1991

The work reported in this thesis was performed in the Department of Paediatrics and Child Health, University of Natal, Durban.

PUBLICATIONS AND CONGRESS PRESENTATIONS

Selected results from this thesis have been published in scientific journals and presented at various congresses. Research workers who were closely associated in the studies are co-authors.

1. Ramkissoon A, Coovadia HM, Loening WEK. A Phase II study of the acellular pertussis vaccine: assessment of antibody responses and side effects in African babies. Paper presented at the 19th International Congress of Paediatrics July 23-28, 1989, Paris, France.
2. Ramkissoon A, Coovadia HM, Loening WEK. Whooping cough - a neglected disease in Southern Africa (Editorial). South African Medical Journal 1989;75(5):560-561.
3. Ramkissoon A, Coovadia HM, Loening WEK, Ndlovana M. Does whole-cell pertussis vaccine protect Black South African Infants? Assessment of post-vaccination events and antibody responses to pertussis toxin, filamentous haemagglutinin and agglutinogens 2,3. South African Medical Journal 1991;79:11;645-649.
4. Ramkissoon A, Coovadia HM, Loening WEK. Whole-cell pertussis vaccine in Black South African infants (Abstract). Clinical Digest Series 1991/2. In press.

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LIST OF ABBREVIATIONS

ACT	Adenylate cyclase toxin
A-DTP	Acellular DTP
AGA	Appropriate-for-gestational-age
AGG2,3	Agglutinogens 2 and 3
AMP	Adenosine monophosphate
AU	Antitoxin units
BCIP	5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt
BCG	Bacille Calmette Guérin
BG	Bordet and Gengou
BSA	Bovine serum albumin
CAMR	Centre for Applied Microbiology and Research
CHO	Chinese hamster ovary
DNA	Deoxyribonucleic acid
DT	Diphtheria-Tetanus
DMF	N,N-dimethylformamide
DTP	Diphtheria, tetanus toxoids, pertussis
ELISA	Enzyme-linked immunosorbent assay
FHA	Filamentous haemagglutinin
EIA	Enzyme immunoassay
GMT	Geometric mean titre
EPI	Expanded programme on immunisation
HLT	Heat labile toxin
HRP	Horseradish peroxidase
ic	Intracerebral
Ig	Immunoglobulin
in	intranasal
kDa	KiloDalton
Lf	Limit flocculation
LPF	Lymphocytosis promoting factor
LPS	Lipopolysaccharide endotoxin
MRC	Medical Research Council
MW	Molecular weight
NBT	p-nitro-blue tetrazolium chloride
NC	Nitrocellulose
OMP	Outer membrane protein
PHLS	Public Health Laboratory Service
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.05% Tween 20
PEM	Protein-energy malnutrition
POD	Peroxidase
PT	Pertussis toxin
SDS	Sodium dodecyl sulphate
SE	Standard error

SGA	Small-for-gestational-age
TCT	Tracheal cytotoxin
TEMED	Tetramethylethylenediamine
TMB	Tetramethyl benzidine
TOPV	Trivalent oral polio vaccine
TST	Tris saline tween
W-DTP	Whole-cell DTP
WHO	World Health Organisation

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SUMMARY

Conventional pertussis vaccine prepared from killed whole cell *B. pertussis* organisms has been in widespread use since the early 1950's. Despite marked reductions in the incidence of pertussis, the use of the vaccine has caused concern because of questions of significant adverse reactions.

Whooping cough is not notifiable in South Africa, and there is consequently a paucity of hard data on efficacy; in addition few cases are proven. Incidence, prevalence, severity and transmission of the disease hence remain a matter of conjecture.

In order to provide background information and determine baseline data for undertaking further studies, available clinical and epidemiological data on whooping cough (pertussis) in South Africa was collated. It was intended to compare the pattern of disease seen in this country with that known in other parts of the world.

Clinical and epidemiological findings from 1525 whooping cough admissions (diagnosed on the basis of clinical criteria) obtained from 6 major infectious disease hospitals around the country for the period 1980-1987/1988 are reported. The data from Durban were available in some detail. Incidence, morbidity and mortality of the disease in South Africa, as elsewhere, was higher in infancy. Infants and young children were predominantly affected, with 31.3% of cases under 12 months of age and 7.2% less than 2 months. Mortality was disproportionately higher in infancy; 53.2% of deaths were in those younger than 12 months of age. There was a slight female preponderance, both in respect of prevalence and mortality. Patients were admitted with pertussis throughout the year, although there was a peak during the winter months (May to October). The typical whoop accompanied the cough in 55.6% of patients. A raised white cell count was recorded in 66% of patients. The most commonly detected complication was bronchopneumonia. Nosocomial infections were frequently encountered.

The average duration of hospital stay was between 10-13 days. Of those with vaccination records, 26% were unvaccinated, 44% had 1 or 2 doses, and 27% had been fully vaccinated with a whole-cell pertussis vaccine combined with diphtheria and tetanus toxoids. The picture, incomplete though it is, reveals a pattern of pertussis similar to that described in other developing countries.

The study reveals huge gaps in our knowledge of this subject in South Africa. More research needs to be done, particularly with respect to improved diagnosis, prevention and treatment; further pertussis should be made a notifiable disease in South Africa.

The whole-cell pertussis vaccine currently used in South Africa has not been adequately evaluated for post-vaccination events and immunogenicity. The development of new purified component pertussis vaccines, which appear to be safer than conventional whole-cell preparations and of equal or almost equal efficacy (although optimal vaccine composition remains to be defined), requires that the concept of early vaccination with this vaccine compared with conventional whole-cell vaccine be examined in order to optimise the immune response to these vaccines in infants; more especially since neonates do not appear to benefit from passive immunity.

Acellular pertussis vaccine has not been evaluated previously in neonates. In order to address the problem of high morbidity and mortality from pertussis in early infancy; and the incorporation of the vaccine into routine vaccination schedules, a phase II trial of acellular and whole-cell vaccines was undertaken in very young infants. The effect of neonatal vaccination with acellular pertussis vaccine on subsequent immunity; and the immunogenicity and short-term safety and reactogenicity of routine primary vaccination with acellular vaccine compared with conventional whole-cell preparations was investigated.

Three hundred and forty-five healthy, full-term African babies were enrolled in the study at birth;

58% of whom were successfully followed for 9 months. Infants were assigned to 1 of 3 vaccine groups in sequence at birth and received either acellular or whole-cell pertussis vaccine combined with D and T (A-DTP or W-DTP) at 2, 4 and 6 months of age. Groups I and II received A-DTP and Group III W-DTP. In addition, at birth, Group I received an additional dose of A-DTP and Group II, a saline placebo injection. No unvaccinated controls were studied for ethical reasons.

Serologic IgG responses to 3 major protective antigens of *B. pertussis*, filamentous haemagglutinin (FHA), pertussis toxin (PT) and fimbrial agglutinogens 2,3 (AGG2,3), were measured by ELISA in sera obtained at birth, and before vaccination at 2, 4 and 6 months and at 9 months of age. The incidence and nature of post-vaccination events were recorded for 14 days after each dose.

A-DTP induced serum IgG responses to PT and FHA comparable with those reported in other studies, with peak PT titres occurring at 6 months of age after 2 doses in babies vaccinated according to the routine schedule (Group II). Surprisingly, response to W-DTP was found merely to restore levels of antibody to PT and FHA to those found in cord blood after 3 doses of vaccine, which questions the immunogenicity of the South African product. Four doses of A-DTP (Group I) did not produce a better antibody response than the 3-dose schedule.

Incidence of all post-vaccination events to both acellular and whole-cell vaccines was low (85.96/1000 doses, 136.36/1000 doses and 76.30/1000 doses in Groups I, II and III respectively). Minor symptoms were more common in recipients of A-DTP, although no significant differences in rates were demonstrated. Neurologic post-vaccination events (without sequelae) occurred more frequently in recipients of W-DTP. No infant vaccinated with A-DTP from 2 months of age (Group II) experienced a neurologic symptom. The risks of major post-vaccination events cannot however be fully quantified in a study of this small size and duration, particularly as the study population has a high incidence of infectious and other

diseases.

A-DTP vaccination commencing at birth produced final antibody titres to PT and FHA which were superior to those of South African whole-cell vaccine but were considerably lower than when the vaccine was incorporated into routine schedules commencing at 2 months of age. The study findings suggest that acellular pertussis vaccines, whether given from birth or from the age of 2 months, appeared safer and produced a better serologic response than the South African whole-cell product which may have impaired immunogenicity.

During the course of the above study, 11 full-term infants with pertussis infection (10 subclinical) were retrospectively diagnosed on the basis of serologic evidence. Of the infants with subclinical disease, all 10 had a ≥4-fold rise in at least 2 different pertussis IgG antibodies and nine had ≥4-fold rise in all 3 IgG antibodies measured. Seven infants had raised IgA antibodies to PT and FHA and 4 infants had raised IgA antibodies to AGG2,3. Subclinical infection provoked antibody production to multiple antigens to differing degrees.

The role of various factors which may have contributed to asymptomatic infection were analysed, viz - household contacts; type of antibody response (clinical vs. subclinical; vaccinated vs. subclinical); maternally acquired antibody levels; vaccination status (number of vaccine doses received); age and gender; and nutritional status.

Special features of the study which require emphasis are: pertussis remained subclinical in unvaccinated babies (most of the subjects were unvaccinated). Subclinical infection followed incomplete primary vaccination with either acellular or whole-cell vaccines commencing at 2 months of age. Subjects were 8 months of age or younger and only 1 had completed primary vaccination. Other infections of infancy were commonly detected; 4 infants had upper respiratory disease about the time of subclinical pertussis. None had a household member with symptomatic pertussis.

Likelihood of subclinical infection was related to significantly lower levels of maternally-acquired pertussis IgG-AGG2,3 antibodies but not associated with infants' nutritional status. Subclinical pertussis is described in very young African babies at an age when the disease is most severe, and therefore has implications for infant morbidity and mortality; it is also relevant to disease surveillance and vaccine-efficacy studies.

Some perinatal factors influencing vaccination were also explored. In this context we looked at:

i. The acquisition of maternal antibodies to *B. pertussis* in paired mother-infant sera from both well-nourished and SGA full-term and pre-term infants, and infants who subsequently developed pertussis infection, and effect of these maternally-acquired antibodies on subsequent antibody responses to acellular pertussis vaccine administered soon after birth, and to acellular and whole-cell vaccines administered from the age of 2 months.

ii. The acquisition of maternal antibodies to diphtheria and tetanus (DT) in paired mother-infant sera from full-term and pre-term infants, and the effect of these maternally-acquired antibodies on serologic responses to neonatal DT vaccination followed by whole-cell DTP vaccination at 2, 4 and 6 months.

Maternal antibodies were measured since little is known about materno-fetal transfer of pertussis antibodies, especially in African countries where inhibition of placental transfer might occur for a variety of reasons. Furthermore, the effect of peri-natal malnutrition and prematurity on transplacental transfer of diphtheria, tetanus and pertussis antibodies has not been conclusively established in these areas.

Significant diphtheria, tetanus and pertussis antibody levels were found in maternal and cord

sera with levels in the latter frequently being higher, indicating active transplacental transfer of antibodies.

The significant pertussis antibody levels in maternal sera were unlikely to be due to the currently used South African whole-cell vaccine (given the poor antibody response to PT and FHA shown in this study). It is assumed that the presence of these antibodies are the end result of natural infection and therefore that pertussis is widespread in the African community.

Maternal and cord IgG-PT and AGG2,3 titres were significantly lower ($p < 0.05$) and maternal IgG-FHA marginally lower ($p \approx 0.05$) in SGA infants compared to cohorts, although placental transfer was equally efficient in both groups. This study has demonstrated that the high titres of maternally derived circulating *B. pertussis* antibodies do not have an inhibitory effect on the subsequent serologic response to acellular vaccine administered in early infancy (2 months) or with the first dose given soon after birth. Protective levels of diphtheria and tetanus antibodies were detected in 100% and 76% of cord sera in pre-term infants and in 85% and 67% of cord sera in full-term infants.

Although the number of infants studied was too small to make a definite comment, there did not appear to be 'tolerance' due to neonatal diphtheria-tetanus vaccination of pre-term infants, or to high levels of maternally-acquired antibody.

During analysis of the data from the phase II study of acellular pertussis vaccines, 25 infants with protein-energy-malnutrition (PEM) were detected on the basis of anthropometric indices. Seventeen infants were small-for-gestational-age (SGA), of whom 9 developed PEM by the age of 9 months. Eight other infants developed post-natal PEM before the completion of the primary vaccination course.

The principal objective of this investigation was to evaluate the reactogenicity and the nature

and extent of immunological impairment, if any, on the serologic responses to acellular vaccine and to conventional whole-cell DTP in SGA infants, and in infants who developed PEM before the completion of the primary vaccination course.

The following indices were evaluated in malnourished infants; (i) anthropometric indices of nutritional status, (ii) intercurrent illnesses including pertussis infection, (iii) post-vaccination events, (iv) serologic responses to vaccination. Results were compared with those obtained in well-nourished (WN) age- and vaccine-matched cohorts.

Overall, peak titres and seroconversion data of all 3 antibodies were not significantly different in malnourished infants though final anti-AGG2,3 titres (at 9 months of age) in Group III were significantly lower ($p = 0.035$).

Although peak and final PT and FHA antibody titres in many SGA and malnourished infants were lower than in WN infants and peak responses were attained at a later age, malnutrition did not significantly affect the response to A-DTP. Peak and final AGG2,3 antibody titres were similar in SGA, malnourished and WN infants. Overall malnourished infants responded no less well to pertussis vaccination than did other vaccinees.

Incidence of minor local and systemic post-vaccination symptoms was not significantly different in PEM and WN groups although induration at injection site and irritability were more frequently reported in the latter. No major neurologic post-vaccination symptoms to either vaccine were reported in SGA infants or infants with PEM at the time of vaccination. No significant differences was noted in the incidence of major symptoms in PEM as compared with WN infants.

One male infant (Group I) who was malnourished at birth and who had been given 2 doses of A-DTP, developed clinical signs of pertussis infection between 2 and 4 months of age. Pertussis

antibody levels immediately prior to infection were not significantly different from those of uninfected age-matched cohorts. The percentage of infants afflicted with common childhood illnesses were similar in PEM and WN groups (46 vs. 43.2%) although the former group incurred significantly more illnesses at an earlier age ($\leq$6 months) ($p = 0.05$, chi square).

These findings, albeit preliminary given the small numbers of subjects studied, suggest that acellular pertussis vaccine may be incorporated into routine vaccination schedules followed in developing countries with the expectation that adequate antibody responses will be provoked in SGA infants and in infants who develop post-natal PEM and that the incidence of vaccine-related adverse effects will be no higher than in WN infants.

Further and more extensive studies are indicated before the use of acellular pertussis vaccines can be recommended for routine primary vaccination of infants in preference to whole-cell preparations in developing countries.

CHAPTER 1

PERTUSSIS: THE DISEASE

1.1 INTRODUCTION

Bordetella pertussis, the causative agent of pertussis (whooping cough) is a small, fastidious Gram-negative non-motile coccobacillus. Pertussis was not differentiated from other respiratory entities with any certainty until 1578 when Guillaume de Baillou described the first epidemic.

Pertussis is a 2-stage disease in which bacterial attachment to the respiratory mucosa is followed by local and systemic disease effects mediated by various toxins and virulence factors which the organism produces. The disease is a highly communicable and often severe respiratory tract infection characterised by paroxysmal coughing, prolonged inspiratory stridor, apnoea, mucus production, vomiting and haemoptysis. Much controversy and discrepancy exists over diagnosis, the relative importance of virulence factors, therapeutic procedures and reporting of morbidity and mortality data. The clinical features of pertussis vary in form and severity with age, immune status and other host factors, and are not easily distinguished.

The disease is distributed worldwide. All ages are vulnerable although morbidity and mortality are highest in infants and children. The severity, duration and incidence of complications tend to decrease with age. Management is primarily directed towards preventing or minimising complications. Prognosis correlates largely with socio-economic conditions.

Adults with modified symptoms are important reservoirs of *B. pertussis*. Subclinical infection occurs in vaccinated individuals exposed to either symptomatic or asymptomatic contacts. Given the variable efficacy of currently used whole-cell pertussis vaccines, the eradication of the disease does not appear to be achievable at present.

1.2 HISTORY

Pertussis (whooping cough) has been known for more than 4 centuries. According to Lapin

(1943) the disease was first mentioned by Moulton in 1540. The first detailed description of a pertussis epidemic was that of Guillaume de Baillou (1536-1616) in his Epidemiorum et Ephemeridum Libri (duo), who recorded an outbreak of the disease in Paris in 1578. In a review of 500 papers on pertussis up to the year 1911, Sticker (cited by Lapin, 1943; Olson, 1975) noted that exhaustive searches for reports of the disease by numerous researchers had failed to uncover references earlier than those given above.

The epidemic nature of the disease was observed in 1682 by Thomas Willis (Garrison, 1921) who also noted that the disease was resistant to treatment with the usual remedies prescribed for other varieties of cough at that time. Between 1670 and 1680 Sydenham (1943) introduced the name 'pertussis' (Latin: per = intensive, tussic = cough). Pertussis had become epidemic in Europe by 1732 and was carried by British seamen to Jamaica, Peru and Mexico (Lapin, 1943). Richard Bright (1789-1858) of Bristol, England was one of the first to describe condensation of the lungs in pertussis (Garrison, 1921).

In 1906 Jules Bordet and Octave Gengou were the first to discover the causal relationship of the whooping cough bacillus, which subsequently became known as the Bordet-Gengou bacillus. This relationship was demonstrated according to Koch's postulate by F.B. Mallory and others in 1913. Grown on a medium rich in blood, the bacterium became known as Haemophilus pertussis. Subsequent demonstration that the X and V factors in blood were unnecessary (Hornibrook, 1940) led to the creation of a new genus *Bordetella* for the causative organism of pertussis.

1.3 *Bordetella pertussis:* MORPHOLOGY, STRUCTURE AND GROWTH

B. pertussis is a minute Gram-negative ovoid coccobacillus 0.2-0.5 μm in diameter and 0.5-1.0 μm in length. The bacteria occur singly, in pairs, or in small clusters; grows best at 36 $^{\circ}$C, and is strictly aerobic. The species is non-motile and non-sporeforming and is killed by exposure to

heat at 55°C for 30 minutes. Fresh isolates have capsules, pili and a rodlike protein called filamentous haemagglutinin.

B. pertussis is a slow-growing organism that requires specialized conditions for growth. Primary media require the addition of fresh blood, albumin, charcoal, starch, or ion-exchange resins to an enriched base medium. The function of the additives is not for nutrition but for adsorption of substances toxic to *B. pertussis* that are normally found in culture media.

Primary isolation from cough plates, supra-laryngeal or per-nasal swabs in the early stages of the disease is possible from glycerine-potato-blood agar (modified Bordet-Gengou medium) to which penicillin has been added. Aerobic incubation for 3-7 days is required for initial growth. Typical colonies are recognisable within 48-72 hours. Colonies are small, raised, glistening, and grey-white in colour. On media containing blood, a narrow zone of haemolysis surrounds each colony. Growth may also be obtained in a suitable enriched broth. Success in isolation depends on the stage of the disease. In the early catarrhal stage before typical symptoms develop the organism can be isolated in almost 100% of cases. This figure drops to between 30-70% in the third week of the disease and thereafter there is a rapid fall to zero.

The organism is a non-invasive respiratory tract parasite of man with a tropism for respiratory ciliated epithelium. It is very susceptible to environmental changes and survives only briefly outside the human respiratory tract under natural conditions.

B. pertussis exists in several distinct serotypes according to their possession of one or more of a set of thermolabile antigens, agglutinogens (AGGs) ie. surface components which react with specific antisera to cause cells to agglutinate (Andersen, 1953; Eldering *et al.*, 1957).

Preston *et al.* (1982) reported AGGs 1, 2 and 3 to be the most important with AGG,1 being common to all strains. When *B. pertussis* is repeatedly subcultured *in vitro*, 4 serologically

distinct antigenic phases: I, II, III and IV develop as a step-wise degradation and are associated with changes in antigenic structure and virulence. Freshly isolated smooth encapsulated strains belong to a single antigenic type designated phase I.Virulent phase I organisms are essential for the preparation of effective vaccine. Unless all antigenic types are adequately represented a vaccine may fail to give protection against all possible infecting strains (Christie, 1987).

Two other organisms, *B. parapertussis* and *B. bronchiseptica* share minor antigenic components with *B. pertussis* and give rise to respiratory infections resembling pertussis.

Phases I and II were considered to be the 'smooth' forms containing toxic and protective activities. Prolonged subculture led to the development of phases III and IV 'rough' forms involving the loss of both virulence and antigenic factors. These changes are genotypic (Leslie & Gardner, 1931).

1.4 PATHOGENESIS

Pertussis is a two-stage disease. The first stage involves infection, ie. the attachment of the organism to human tracheobronchial ciliated epithelium (which permits it to resist normal host clearance mechanisms), and subsequent growth and multiplication (colonization). The second stage involves the production of local disease effects (ciliostasis and sloughing of cilia and ciliated epithelial cells) and systemic disease effects (including lymphocytosis and alterations in blood chemistry) and is mediated by various toxins of *B. pertussis*.

Following inhalation of infected droplets, virulent phase I *B. pertussis* bacteria attach to the ciliated tracheobronchial epithelium. The bacteria are not invasive and do not infect submucosal or other sites in the body. Throughout the course of the disease they adhere to neuraminic acid or similar receptors in the superficial epithelial layers of the respiratory tract

and bronchial tree, hence causing local damage, ie. inflammatory changes and pathological lesions. The specificity of this attachment is attributable to the production of exotoxins, especially pertussis toxin (PT) and to two adhesins, filamentous haemagglutinin (FHA) and bacterial agglutinogens (Tuomanen *et al* ., 1983; Christie, 1987; Zhang *et al* ., 1985). The presence of both PT and FHA seem to be required for effective adherence to tracheal cilia, however the nature of the mechanism is unknown. The ability to adhere is important to the virulence of the organism and protection from disease may arise in part from protection against adherence.

Together with PT, a second toxin of *B. pertussis*, adenylate cyclase toxin (ACT) adversely affect the host immune effecter cell function and thereby contribute to the evasion of host defences and thus the growth and multiplication of the bacteria and the propagation of infection.

Following attachment, *B. pertussis* overcomes the nonspecific clearance mechanisms of the ciliated cells by the action of a third toxin, tracheal cytotoxin (TCT), a small glycoprotein toxin which allows infection to persist by disrupting normal clearance mechanisms (Weiss & Hewlett, 1986). Ciliary activity is hence diminished and bacteria, mucus, leucocytes and exudate accumulate in the respiratory tract, resulting in bronchial obstruction and frequently in patchy areas of atelectasis and emphysema. The nonciliated cells are not involved and maintain the integrity of the epithelial lining.

Local tissue damage caused to the respiratory epithelium probably explains the long duration of whooping cough symptoms. Animal studies suggest that TCT, dermonecrotic toxin lipopolysaccharides, ACT and haemolysin all play a role in local damage.

The causes of systemic disease effects (paroxysmal cough, lymphocytosis and leucocytosis, encephalopathy) are uncertain. They may be a result of the tenacious nature of secretions together with a loss in ciliary action, local irritation or the central effect of blood-borne toxins

and metabolic factors. The coughing spasms do not disappear when the organism disappears (eg. as a result of antibiotic treatment) suggesting some central mechanism.

1.5 VIRULENCE FACTORS OF *B. pertussis*

Several proteins, both toxins and adhesins have been found to be important in the pathogenesis of clinical manifestations of the disease. Figure 1.1 shows a scheme of events in which the clinical stages of the disease are explained on the basis of the virulence factors involved.

1.5.1 TOXINS

These include pertussis toxin (PT), heat-labile toxin (HLT), adenylate cyclase (ACT), tracheal cytotoxin (TCT) and lipopolysaccharide endotoxin (LPS). Toxic activities have been demonstrated mainly in animal models, in some of which the pathogenesis and immune responses are demonstrably different from those in man.

i) Lymphocytosis or leucocytosis-promoting factor or pertussis toxin (PT): PT is an exotoxin of molecular weight 117000. It appears as spherical structures 6 nm in diameter by electron microscopy (Irons & MacLennan, 1979; Askelöf & Gillenius, 1982; Morse & Morse, 19767). It is found both cell bound and cell-free (in cultures of *B. pertussis*). PT contains a pentameric protein composed of five dissimilar subunits of decreasing molecular size termed S_1-S_5. The S_1 subunit is endowed with enzymatic activity while the remaining subunits are target-cell receptor-binding subunits that allow S_1 to enter host cells.

PT is the most important toxin produced by *B. pertussis* and is believed to play a major role in the pathogenesis of, and immunity to pertussis (Pittman, 1979,1984). It is known to play a role in attachment and acts as a binding mediator between the toxin and the cell membrane (Tuomanen & Weiss, 1985). Antibodies to PT prevent infection in mice as well as adherence of

bacteria to cilia in mice (Tuomanen, 1985).

PT is believed to be responsible for most of the systemic manifestations of *B. pertussis*. It is not directly cytotoxic, but stimulates the adenylate cyclase system by modifying cyclic AMP of host-cells. It also promotes leucocytosis and lymphocytosis, increases susceptibility to histamine, and causes pancreatic islet cell activation when injected into laboratory animals (Oda *et al* ., 1984; Pittman, 1984; Hewlett, 1984). The role of PT in local injury, cough, or the prolonged course of the disease is unclear.

PT also affects macrophages (Levine & Sowinski, 1972; Meade *et al* ., 1984,1985; Vogel *et al* ., 1985); neutrophils (Becker *et al* ., 1986) and lymphocytes (Morse & Morse, 1976; Spangrude *et al* ., 1984) in ways that aid survival and multiplication of *B. pertussis* on host respiratory epithelium. PT prevents the migration of lymphocytes and macrophages to areas of inflammation and interferes with secretion of certain neutrophil enzymes required in phagocytosis. Figure 1.2 shows an electron micrograph of PT molecules.

ii) Dermonecrotic heat-labile toxin (HLT): This was the first *B. pertussis* toxin to be reported and purified (Banerjea & Munoz, 1962). HLT is a cell-associated protein toxin of molecular weight (MW) 102000. It causes dermal necrosis, splenic atrophy and death when injected into mice (Kurokawa *et al* ., 1969; Cowell *et al* ., 1979) and is produced by all freshly isolated strains of *B. pertussis*.

HLT seems not to be required for the prophylaxis of whooping cough but may play an essential part in the host-parasite relationship. Since its discovery by Bordet & Gengou in 1909, the toxin has been considered to be responsible for inflammatory changes in the respiratory tract mucosa that may contribute to the characteristic clinical features of pertussis.

As most studies have been done with impure toxin, there is no definite known role for HLT in the

pathogenesis of whooping cough (Munoz, 1971; Olson, 1975; Wardlaw & Parton, 1983). Recent findings of the vasoconstrictive effect of HLT on the peripheral blood supply to the respiratory tract have suggested that it may be involved in the initial stages of the disease (Endoh *et al* ., 1986).

iii) Adenylate cyclases (ACT): *B. pertussis* has two distinct ACT complexes, one extracytoplasmic and one intracellular. These were discovered by Wolff & Cooke (1973). The exact role of ACT in the immunology and pathology of pertussis is not clearly elucidated. ACT has a nonspecific effect on the host, causing fever and inflammation. It has been shown to interfere with chemotaxis and superoxide production by polymorphonuclear leucocytes. It also catalyses cyclic AMP (cAMP) production from endogenous ATP. The accumulation of cAMP inhibits the activities of phagocytic cells involved in host defence (Confer & Eaton, 1982; Hewlett, 1984; Friedman *et al* ., 1987; Pearson *et al* ., 1987). The toxin is activated within the cell by calmodulin, a eukaryotic regulatory protein. The presence of ACT is hence conducive to secondary bacterial colonisation and infection, resulting in the fatal complications of pertussis. It has been postulated that the synthesis of the various other toxins of *B. pertussis* is controlled and directed by ACT activity (Stainer, 1988).

iv) Tracheal cytotoxin (TCT): TCT, a small glycoprotein toxin was discovered by Goldman *et al* . (1982). Its direct role in inducing paralysis of cilia of the respiratory epithelium and in the inhibition of DNA synthesis has been experimentally demonstrated in hamster tracheal cell cultures. This interference with ciliary activity (and subsequent loss of a major defence mechanism in the respiratory tract) may contribute to secondary infections.

v) Lipopolysaccharide endotoxin (LPS): LPS is a heat-stable envelope toxin present in all Gram-negative bacteria, including *B. pertussis* (LeDur *et al* ., 1980). *B. pertussis* LPS is composed of two distinct factors, LPS-I and LPS-II. LPS is thought to inhibit chemotaxis of the macrophage, thus inducing the infectious process.

Its inclusion in whole-cell pertussis vaccines may contribute to their reactogenicity; when present in high concentrations LPS is thought to contribute to post-vaccination pyrexia. Steps have hence been taken to minimise LPS levels in the development of new acellular vaccines. No evidence of the effects of LPS are apparent in clinical pertussis.

1.5.2 ADHESINS

These are 'outer membrane proteins' or components of the bacterial surface which play a role in the attachment of the bacteria to host cells and hence initiate infection.

i) Agglutinogens (AGGs): *B. pertussis* AGGs were first recognised by Andersen (1953) and Eldering *et al* . (1957). AGGs are non-toxic serotype specific bacterial surface protein antigens numbered one through seven and are used to serotype pertussis (Carter & Preston, 1984; Ashworth *et al* ., 1985; Irons *et al* ., 1985; Zhang *et al* ., 1985; Cowell *et al* ., 1987). Although AGGs are protective antigens in mice they do not appear to play a major role in adherence of *B. pertussis* to human ciliated upper respiratory tract epithelia.

Three major AGGs (1, 2 and 3) are found in varying combinations in different pertussis strains, thereby differentiating four widely recognised serotypes (1,2,3; 1,2; 1,3; 1). Only the first three of these infect man (Preston & Stanbridge, 1972), suggesting an important role for AGG2 and 3 in virulence and in prophylaxis. Current whole-cell vaccines are being developed to contain AGGs 1, 2 and 3 (WHO, 1964).

ii) Filamentous haemagglutinin (FHA): FHA is a filamentous cell-surface protein, MW 130,000, consisting of short and thin filaments about 2 nm in diameter and 40-100 nm in length (Arai & Sato, 1976; Morse & Morse, 1976; Pittman, 1984). FHA is a non-toxic antigen and has haemagglutinating activity. Adherence is the only known function of FHA. As supported by experiments in rabbits and mice, it plays a role in attachment of *B. pertussis* to ciliated epithelia

and hence in initial colonisation of the respiratory tract (Robinson *et al*., 1985; Urisu *et al*., 1986; Arai & Sato, 1976). Figure 1.2 shows an electron micrograph of FHA molecules.

iii) 69kDa outer membrane protein: This is a non-fimbrial 69 kilodalton (kDa) agglutinogen which is present in all virulent strains of *B. pertussis* and an antigenically similar protein is found on closely related species, *B. parapertussis* and *B. bronchiseptica* (Brennan *et al*., 1988). The 69kDa OMP has been shown to stimulate production of agglutinating antibodies in mice.

1.6 DISEASE

1.6.1 CLINICAL MANIFESTATIONS

The clinical course of the disease classically occurs in four stages and is not altered appreciably by the administration of specific microbial agents. There are many descriptions of classic disease (Lapin, 1943; Olson, 1975; Christie, 1987; Linnemann, 1986).

i) Incubation period: This stage may vary from 5-21 days (usually 7-10 days) and follows inhalation of infected droplets. It is not associated with any specific symptoms. After incubation, pertussis follows a prolonged course consisting of 3 overlapping stages: catarrhal, paroxysmal coughing and convalescence.

ii) Catarrhal (prodromal) stage: This stage is characterised by symptoms of a mild upper respiratory tract infection or common cold (low grade fever, rhinorrhea, sneezing, nasal catarrh, short dry nocturnal cough) which lasts for about 1-2 weeks (Krugman *et al*., 1985). Symptoms are indistinguishable from disease due to other respiratory pathogens. In the absence of a history of contact, whooping cough is not suspected.

The disease is most communicable at this stage, as large numbers of organisms are present in the nasopharynx and the mucoid secretions. The risk of transmission decreases through subsequent stages. This is the only stage of the disease at which antibiotics may be helpful in

shortening duration.

iii) Paroxysmal stage: This stage lasts for 4-6 weeks but may continue even longer. It is characterised by progression from mild to severe paroxysmal coughing, forceful inspiration, characteristic high-pitched whoop and vomiting. Coughing paroxysms vary in frequency from 3-4 times a day (mild cases) to 6-7 times an hour (severe cases). The characteristic inspiratory whoop follows a series of coughs as air is rapidly drawn through the narrowed glottis. During an attack the child's face becomes red or cyanotic, the tongue protrudes and facial oedema, particularly around the eyes may occur. Haemoptysis may also occur. Body temperature remains normal unless a complication such as secondary atelectasis or bronchopneumonia occurs. Paroxysms often end in mucus production and vomiting. The cough is more common at night and is precipitated by eating, drinking or crying. This stage is frequently associated with marked leucotytosis, primarily as a result of increased levels of circulating lymphocytes. Absolute lymphocyte counts of 12,000=100,000 mm^3 are typical. Most complications and deaths occur during this paroxysmal stage.

iv) Convalescent stage: This stage lasts 3-4 weeks and is marked by a decrease in frequency and severity and eventual cessation of the paroxysmal cough. Other features of the disease gradually fade as well. Patients are usually free of *B. pertussis* organisms during convalescence despite persistent coughing for several months after onset of the disease. Relapse may be precipitated by subsequent intercurrent viral upper respiratory tract infections for up to one year after the disease.

1.6.2 DIAGNOSIS AND DIFFICULTIES

Clinical diagnostic criteria: The clinical features characteristic of pertussis are paroxysmal coughing following by a high-pitched inspiratory whoop; vomiting and/or choking, the production of sticky stringy mucus; red or cyanotic appearance; periorbital oedema, subconjunctival haemorrhage and epistaxis.

If the clinical features described above are present, the diagnosis of pertussis is rarely in doubt. Pertussis is however no longer a disease with universally typical clinical features. Changing socio-economic factors, vaccination and advances in the management of complications are constantly modifying the illness.

Clinical disease manifests itself in varying forms and degrees of severity, ranging from subclinical disease or mild respiratory illness to severe illness with sequelae. Symptoms vary with age, immune status and the general health of the individual which makes difficult a diagnosis purely on clinical grounds. The illness is often mild and atypical, especially in older children and adults, in younger children who have been incompletely vaccinated and in very young children who may be partially protected by maternally derived antibody (Preston, 1977; Cherry *et al* ., 1988).

Several investigators have suggested that pertussis vaccination may modify the severity of infection even if full protection is not achieved (Cherry *et al* ., 1988; Romanus *et al* ., 1987; Lambert, 1965; *Ad hoc* Group for the Study of Pertussis Vaccines, 1988). The occurrence and magnitude of subclinical *B. pertussis* infection in vaccinated populations are unknown. One-third to two-thirds of infection in vaccinated contacts is thought to be subclinical (Mertsola *et al*., 1983; Steketee *et al* ., 1988; Fisher *et al* ., 1989; Long *et al* ., 1990b; Zackrisson *et al* ., 1990).

Gordon & Hood (1951) suggested that atypical or abortive pertussis constitutes about 25% of all pertussis cases. In an outbreak in the United States only 27% of patients had respiratory symptoms (Fisher *et al* ., 1989). Most studies have shown that <50% of infected children consistently whoop. Very young infants may present only with apnoea or cyanosis, the whoop may be absent and paroxysms may be less frequent. Without a history of contact it is often impossible to diagnose the disease on clinical grounds alone in these infants.

A cough suggestive of pertussis may occur in infants with bronchiolitis, bronchopneumonia, cystic fibrosis or chlamydial pneumonitis. In older children a similar cough may accompany interstitial pneumonia, inhalation of a foreign body or pressure on the trachea by enlarged tracheobronchial glands. Infections with *B. parapertussis, B. bronchiseptica, Haemophilus influenzae, Mycoplasma pneumoniae, Chlamydia trachomatis* and adenoviruses also produce paroxysmal coughing (Connor, 1970; Nelson, 1975; Granström & Askelöf, 1982).

Laboratory diagnosis: Diagnostic tests for pertussis include lymphocyte counts, x-ray, culture and various serologic tests. The latter are not routinely used and are not easily applicable in the field or even at hospitals in the Third World, and often produce ambiguous results. The difficulties in diagnosing pertussis on the basis of clinical recognition are hence compounded by inadequacies in laboratory confirmation. No single laboratory investigation has been shown to be both highly sensitive and highly specific in the diagnosis of pertussis (Granström *et al* ., 1982; Steketee *et al* ., 1988; Onorato & Wassilak, 1987).

A total lymphocyte count in peripheral blood which is raised above normal for the age of the patient, or a markedly increased proportion of lymphocytes, supports a clinical diagnosis (Brooksaler & Buchanan, 1976). A leucocytosis of > 10,000 mm^3 (sometimes rising to 100,000 mm^3) with a relative (60%) lymphocytosis may occur from the second to fifth week of disease. The absence of leucocytosis however does not exclude diagnosis. It is widely recognised that many cases of true pertussis do not develop a significant increase in circulating lymphocytes; also the many causes of lymphocytosis ensure that a positive result lacks diagnostic specificity.

Chest radiographs are clear in uncomplicated pertussis. In severe cases respiratory complications, segmental or lobar collapse, atelectasis and enlargement of mediastinal lymph nodes may be apparent.

Culture of Bordetella from nasopharyngeal swabs or cough plates (Bradford & Slavin, 1940) is

considered the only reliable method of laboratory diagnosis (Abbott *et al* ., 1982). Culture is however difficult because *B. pertussis* has a tissue tropism that ensures that specimens are contaminated with faster growing organisms, fastidious growth requirements, and rapid death in transport. In addition, the organism is only present in large numbers in the nasopharyngeal sections during the earliest catarrhal stages of the disease when symptoms are so nonspecific that the diagnosis is rarely considered (Darling, 1983; Linnemann, 1979). Frequency of isolation decreases with duration of the disease, even in the presence of clinical symptoms. Under optimal conditions, most series have been unable to culture the organism from >50% of cases (Walker *et al* ., 1981; Granström *et al*., 1982; Fisher *et al* ., 1989, Halperin *et al* ., 1989); diagnosis thus rests heavily on clinical recognition.

Serologic diagnosis of pertussis involves direct immuno-fluorescent antibody staining of bacteria in nasopharyngeal secretions or the detection of specific pertussis antibodies in serum and secretions by direct agglutination, haemagglutination, or complement-fixation (Combined Scottish Study, 1970; Abbot *et al* ., 1971; Macaulay, 1979; Winter, 1953; Bradstreet *et al* ., 1972). Several ELISA techniques have also been described with varying results.

As with bacterial culture, a negative result in the various serologic tests does not exclude pertussis. More importantly, a positive result is ambiguous because of antigenic sharing, and hence cross-reactivity with other bacteria, viz. *B. bronchiseptica, B. parapertussis, Haemophilus,*and *Staphylococcus* (Hörby *et al*., 1976; MacLennan *et al* ., 1977).

Serologic diagnosis is confounded by pertussis antibodies from previous vaccination and by age-related differences in antibody response to disease. In addition, the tests depend on the demonstration of a rise in titre over time. Their usefulness in early diagnosis is hence limited (Mertsola *et al* ., 1983; Granström *et al* ., 1982; Nagel & Poots-Scholten, 1983).

The optimum protocol for laboratory diagnosis of pertussis in all forms and at all stages is

probably a combination of culture, immunofluorescence and serology.

In an effort to improve the reliability of pertussis data the EPI has suggested the following criteria for diagnosis (Expanded Programme on Immunisation, 1983) :

1. Suspect whooping cough:

   a. History of severe cough, and
   b. History of any one of the following -
      i. cough persisting two or more weeks;
      ii. fits of coughing, and
      iii. cough followed by vomiting.

2. Probable whooping cough:

   a. Suspect case, and
   b. Any one of the following -
      i. typical findings on physical examination by a qualified health worker. In young infants prolonged coughing followed by a period of apnoea and cyanosis, coughing followed by a typical breath intake and 'whoop', fits of severe coughing, coughing followed by vomiting, subconjunctival haemorrhages;
      ii. exposed to a suspect case in the previous three weeks (incubation period usually 7-10 days);
      iii. epidemic of whooping cough in the area, and
      iv. white blood cell count with 15,000 lymphocytes per mm^3 or more.

3. Confirmed whooping cough:

   a. Probable case, and
   b. Positive culture or immunofluorescence of nasopharyngeal secretions for *B. pertussis* bacteria.

1.6.3 COMPLICATIONS

Neurological complications: Major neurologic problems and encephalopathy occurs in 0.1-4% of whooping cough cases. This is due either to toxic or asphyxial brain damage or to intracranial haemorrhage provoked by coughing spasms and may lead to many temporary or

permanent deficits. These range from fine tremors occurring with the cough to generalised seizures or convulsions, coma, mental retardation, cranial nerve palsies, hemiplegia, ataxia, aphasia, blindness and deafness. Paralysis is a fairly frequent sequel and is most often hemiplegic.

The risk of serious acute brain damage after whooping cough infection has been shown to be six times that after three doses of whole-cell DTP vaccine. The rate of neurological complications following the disease as compared with that following pertussis vaccination is presented in Table 2.1. The incidence of encephalopathy in one large series of hospitalised children was 1% (Litvak, 1948). Incidence of complications and prognosis are inversely proportional to age; the younger the child the more likely are permanent neurologic sequelae.

Apnoeic attacks or flaccidity and loss of consciousness are common in young infants and may represent atypical convulsions. One third of children in whom encephalopathy develops die (death occurs most frequently in those younger than six months and in the debilitated); one third survive with sequelae (mental retardation, focal paralysis, changes in personality and behaviour); and one third appear normal (Zellweger, 1959).

Respiratory complications: Rapid inspiration (causing the whoops) may result in aspiration of respiratory secretions and encourage the development of bronchopneumonia and/or lobar or segmental collapse, the two most common complications of whooping cough (Christie, 1987).

Bronchopneumonia and bronchiolitis are responsible for most deaths under three years of age and is due to bacterial or viral superinfection of the lower respiratory tract with, eg. *Pneumococcus, Haemophulus influenzae* or *haemolytic Streptococcus*. The incidence of bronchopneumonia during pertussis appears to be similar for all ages under the age of five years but it may be more severe in infants (British Society for the Study of Infection, 1970). Bronchiectasis will occur in persistent atelectatic segments and may be a long-term

consequence of overdilation of the airways. Atelectasis is common but may only be detected by chest radiography. Infrequently, subcutaneous and interstitial emphysema or pneumothorax follow rupture of alveoli during violent coughing.

Sudden death from asphyxia due to obstruction of the airways may occur in uncomplicated cases. There is a suggestion that lung function may be impaired and that there may be enhanced susceptibility to other respiratory infections for some years following pertussis (Swansea Research Unit, 1985). The onset of asthma and the activation of latent tuberculosis has been observed to follow attacks of pertussis. Otitis media is common.

Cardiac complications: Congestive cardiac failure may occur in a few cases with severe pneumonia. Myocarditis due to bacterial exotoxin may also occur.

Secondary pressure effects accompanying severe pertussis: Increased venous pressure and congestion during coughing paroxysms can cause facial petechia, melena, subdural and spinal haematoma, subconjunctival and occasionally intracranial haemorrhages, epistaxis, and anoxic brain damage. Figure 1.3 shows a child with subconjunctival haemorrhage during subsiding pertussis. Raised intrathoracic and intra-abdominal pressure during paroxysms may cause umbilical and inguinal herniae (especially in the very young when closure of the peritoneal sacs is incomplete) and rectal prolapse. In those with lower incisor teeth, forceful extrusion of the tongue during coughing can result in persistent ulceration of the frenulum under the tongue.

Intercurrent illness: The long duration of whooping cough provides opportunity for other common infections such as gastroenteritis, childhood exanthemas and measles. Loss of weight and dehydration may present serious problems.

1.6.4 TRANSMISSION

Pertussis is highly contagious. Transmission approaches 100% when introduced into a

susceptible population (Biellik *et al* ., 1988; Broome *et al* ., 1981; Mertsola *et al* ., 1983). The disease is spread by respiratory droplets, either directly inhaled or via contact with contaminated surfaces. The intensity of transmission is affected by the intimacy and frequency of exposure as well as by vaccination status (Fine, 1988). The only known host of the disease and reservoir of infection is man. There is no evidence that animals play a part in disease transmission (Kendrick, 1975).

There is evidence of contagious spread of *B. pertussis* to vaccinated individuals (Long, 1990a). The explosive expiratory paroxysms characteristic of pertussis provide an excellent mechanism for transmission of infection to close contacts. Unless an infected child is isolated from the early catarrhal stage of the illness, most household contacts will be susceptible. It has been estimated that one case will infect from 55-100% of non-immune susceptible contacts (Pittman, 1984) and up to 50% of non-immune school colleagues (Gordon & Hood, 1951; Kendrick, 1975). The period of communicability extends from seven days after exposure to three weeks after onset of paroxysms. There is evidence that younger children shed more organisms, and for longer periods, sometimes two to three months (Combined Scottish Study, 1970).

There is evidence of silent transmission of *B. pertussis* (Long *et al* ., 1990a; Jenkinson & Pepper, 1986; Krantz *et al* ., 1986). The importance of individuals who are asymptomatic or mildly symptomatic in spread of infection is incompletely understood. Duration of culture positivity and degree of contagiousness are greatest in individuals with typical pertussis.

In the past children with pertussis were thought to be the source of family spread. Family studies now show that older vaccinated persons with modified illness are likely to serve as primary cases and the unvaccinated children with whooping cough, the secondary cases (Nelson, 1978; Bass & Stephenson, 1987).

Several factors are involved in controlling transmission of *B. pertussis*, viz. early recognition

and isolation of infectious patients, use of antimicrobials to eliminate organisms from the nasopharynx and vaccination.

1.6.5 MANAGEMENT

Management is primarily directed towards preventing or minimising complications. Most cases are mild and the patient can usually be cared for at home. Severe disease, especially in infants, however requires close nursing supervision to prevent inhalation of secretions and vomitus during spasms. Use of oxygen for infants with cyanosis and/or dyspnoea may be required. Isolation of infected patients from non-immune individuals is advisable, especially during the infectious period. Once illness is established management should concentrate on comforting the patient. There should be avoidance of factors which precipitate paroxysms, eg. sudden noise. Physiotherapy may help reverse or prevent pulmonary atelectasis or bronchopneumonia but must be carried out so as not to precipitate further spasms. Frequent vomiting may affect nutrition. Small feeds, if necessary by nasogastric tube, may reduce frequency of vomiting.

Immunoprophylaxis: The use of vaccines after onset of symptoms has not been shown to be effective and is not recommended. Human hyperimmune pertussis immunoglobulin is not recommended post-exposure and has no effect in the paroxysmal disease stage. Recently-infected infants or those in imminent danger of exposure may be protected to some extent by pertussis Ig with a high anti-PT titre (Johnsson & Lundström, 1950; Hansen, 1966).

Drug therapy: A wide range of drugs have been used for the symptomatic treatment of whooping cough: anti-tussives, sedatives, anti-spasmodics, antibiotics, bronchodilators, and mucolytics. Early recognition and early antibiotic therapy (ampicillin, tetracycline, chloramphenicol, co-trimoxazole, erythromycin) may help to reduce the duration and severity of the infection. Erythromycin is preferred because of its clinical effectiveness and relative lack of toxicity. The efficacy of erythromycin prophylaxis in controlling spread of *B. pertussis* has varied (Fisher *et al* ., 1989; Steketee *et al* ., 1988, Halsey *et al* ., 1980; Bass, 1983). Early

treatment with antibiotics has been found to modify or shorten the clinical course of the disease (Bass *et al* ., 1969; Altemeier & Ayoub, 1977), however the normal course of the disease is not altered if even slight symptoms have been present for longer than one week (Medical Research Council Report, 1953). Antimicrobial therapy is useful for limiting spread to other susceptible individuals. Erythromycin prophylaxis is recommended for all asymptomatic non-immune household contacts, moreso those under the age of two months. Neonatal dosage should be given at 5 mg/kg body weight/24 hours in four divided doses for two weeks and salbutamol 0.5 mg/kg/day in three divided doses.

There is evidence that cortico-steroids are effective in reducing paroxysms and shortening the course of the disease; the dosage of prednisone is 5-20 mg/kg/day for one week (Coovadia & Loening, 1988). The potential side effects and the possible need for repeated courses must be taken into account (Zoumboulakis *et al* ., 1973).

Simple cough sedatives are unhelpful and may encourage retention of respiratory secretions (Miller & Fletcher, 1987). Salbutamol as a cough suppressant has been studied in a few small trials (Peltola & Michelsson, 1982; Krantz *et al* ., 1985). There may be some control (not cure) of the cough with this drug but the indications for its use in pertussis are yet to be defined (Walker *et al*, 1988).

1.6.6 PROGNOSIS

Pertussis causes prolonged morbidity, lasting several weeks or months. Complications worsen the prognosis and these occur most frequently in infancy. Prognosis is inversely proportional to age. About 10% of all cases and 70% of all deaths occur in the first year of life. In one report eight of ten deaths were in infants under six months old (Miller & Fletcher, 1976), and in another, ten of twelve deaths (British Society for the Study of Infection, 1977). Over the age of four to five years complications are rare and the disease carries a good prognosis.

The greater availability of intensive care facilities in developed countries, improved nourishment and housing, improved control of secondary pulmonary infections and reduction in severity of disease due to vaccination have combined to reduce mortality and hence prognosis, particularly in those less than one year of age.

1.7 EPIDEMIOLOGY

1.7.1 GEOGRAPHICAL EXTENT OF PERTUSSIS

Pertussis exists and persists worldwide in all continents except for some remote or island populations (Cooper & Fitch, 1983) and continues to be a major cause of morbidity and mortality. The WHO Expanded Programme on Immunisation (EPI) estimates the total number of cases of pertussis worldwide to be 60 million annually with half to one million deaths (Muller *et al*., 1986). Geographic distribution of these cases is assessed largely on routine morbidity and mortality data, the reliability and reporting efficiency of which is generally low. Records thus give only a rough indication of prevalence. Underdiagnosis of the disease, particularly if the classic symptoms have not occurred, is also thought to contribute to underestimation of the reported incidence of the disease (Cherry *et al*., 1988). A study in the United Kingdom (Fine & Clarkson, 1985) concluded that only 5% to 25% of pertussis infections were reported in England and Wales over the past 30 years.

In developing countries the great majority of cases are not likely to be reported because they do not come into contact with the health services, nevertheless >50,000 deaths due to pertussis are reported annually. In many countries the disease is not notifiable and reliable statistics therefore not available.

Epidemic waves occurring in cycles of three to five years have been noted in England, Wales, Kenya and Canada in both pre-vaccine and vaccine eras regardless of vaccine coverage (Muller *et al*., 1984; Fine & Clarkson, 1982, 1986b; Fine, 1984; Halperin *et al*., 1989).

1.7.2 MORBIDITY AND MORTALITY

Although all ages are vulnerable to pertussis, the disease primarily affects infants and young children. About 65% of cases occur under the age of seven years. The epidemiology of pertussis is unusual in that infants receive no effective placentally transferred immunity from their mother (Phillips, 1921) and are susceptible from birth. The highest incidence rates are observed in developing countries where vaccination coverage is low (Morley, 1966). WHO has estimated that in countries without a vaccination programme 80% of surviving newborns will acquire pertussis in the first five years of life (Fine, 1984) and that 2% of all newborns in developing countries will die of pertussis (Galazka *et al* ., 1984; Voorhoeve *et al* ., 1978; Joint Committee on Vaccination and Immunisation, 1981). Vaccination does not give complete protection from disease but vaccinated children seldom have an illness severe enough to warrant hospital admission (Walker *et al*., 1981).

WHO sources estimate 60,000 deaths due to pertussis in Africa annually (WHO, 1988). Case-fatality is age-related. In the majority of recent studies the disease was most severe, least easily diagnosable and carried the highest mortality in infants aged under one year in both developing and developed countries (Nelson 1978; PHLS Communicable Disease Surveillance Centre, 1983; Halperin *et al* ., 1989; Broome *et al* ., 1981; Biellik *et al* ., 1988). This is partly because infants experience more serious complications and also because of incomplete vaccination at this early age. Reported case-fatality rates (CFRs) are usually based on hospital figures and are therefore inflated since only serious cases are hospitalised. Figures range from 0.5 per 1000 in the United Kingdom to almost 14% in Uganda (Bwibo, 1971). Unrecognised post-perinatal mortality from pertussis was found to be substantially greater than certified (Nicoll & Gardner, 1988). The long duration of the illness allows opportunities for other common infections to occur. These combined illnesses make it difficult to determine the precise contribution of pertussis to death rates.

Unlike many childhood infections where the reverse is the case, the attack rate in females

slightly exceeds that in males and is associated with increased severity and a higher fatality rate in all age groups except those younger than one year (Cherry *et al*., 1984; Walker *et al*., 1981).

Morbidity is the same in urban and rural communities and whether epidemic or endemic conditions prevail. Lower socio-economic status increases both incidence and complications of pertussis. Increased mortality has been shown to correlate with crowding, a low level of family education and a greater number of small children in the household (Gordon & Hood, 1951). Severe epidemics tend to occur where the standard of general health and nutrition is poor.

No relationship has been established between pre-existing malnutrtion and severity of pertussis (Muller *et al*., 1987). The CFR is highest in pre-weaned infants at an age when weight gain is usually satisfactory. However improved socio-economic conditions have led to a decline in the incidence and fatality rate in many developed countries. The resurgence of pertussis in the United Kingdom, Sweden and Japan in the 1970s following controversy over the safety of vaccination showed the disease to be capable of re-establishing itself in countries without the nutritional and hygiene problems of the Third World.

1.8 PROSPECTS FOR ERADICATION

Although eradication of pertussis is theoretically possible through extremely high coverage of young infants with highly effective vaccines, the biology of the disease mitigates against this. Several characteristics of the disease favour continued circulation of the organism, causing disease in susceptible individuals:

i. Infants <6 months old are susceptible prior to the age when the three doses of vaccine thought to be necessary for protection can be administered (Cherry *et al*.,

1988). The necessity for multiple doses of vaccine delay the appearance of immunity.

ii. The organism from infected individuals is highly contagious to susceptible infants. Household secondary attack rates reported over 80% in several studies (Noble *et al.*, 1987).

iii. Comprehensive surveillance and epidemic control is difficult since clinical expression may be attenuated or atypical and hence diagnosis is difficult. Failure to recognise cases permits spread through several successive individuals and over great distances before outbreaks are detected.

iv. Older children, adolescents and adults are susceptible because vaccine efficacy decreases with increasing time intervals after a booster dose (Lambert, 1965).

v. The circulation and prevalence of *B. pertussis* has been unchanged by vaccination.

Analysis of data from several populations suggest that at least 94% of a population must be immune in order for pertussis to disappear spontaneously as a result of herd immunity (Anderson & May, 1985). Elimination has not yet been demonstrated in any country. In Canada a high rate of pertussis infection continues despite vaccination rates of >95% (Halperin *et al.*, 1989).

The current rate-limiting factor is the incomplete protection provided by the contemporary whole-cell vaccine. Given the variable efficacy of this vaccine, disease transmission would not be stopped even if vaccine coverage was 100%.

Poor vaccination rates either due to fears of possible toxicity in developed countries or to inadequate facilities in developing countries contribute to difficulties in controlling the disease. According to Cook (1979) >95% of all pertussis deaths occur in developing countries. It is estimated that 0.6 million deaths can be prevented yearly if a full course of DTP is given (WHO, 1990). Vaccine coverage rates for DTP (of 12 month olds) ranged from 34% in Africa to 71% in developed countries in 1988. Momentum on vaccination is increasing and 75% coverage is

aimed for in poor countries by the year 2000.

The elimination of pertussis appears at present not to be an achievable goal (Fine, 1988). The availability of an improved vaccine with efficacy consistently over 90%, requiring only one or two doses, and less reactogenic than current whole-cell vaccines as well as improved methods of diagnosis are pre-requisites for future eradication of the disease.

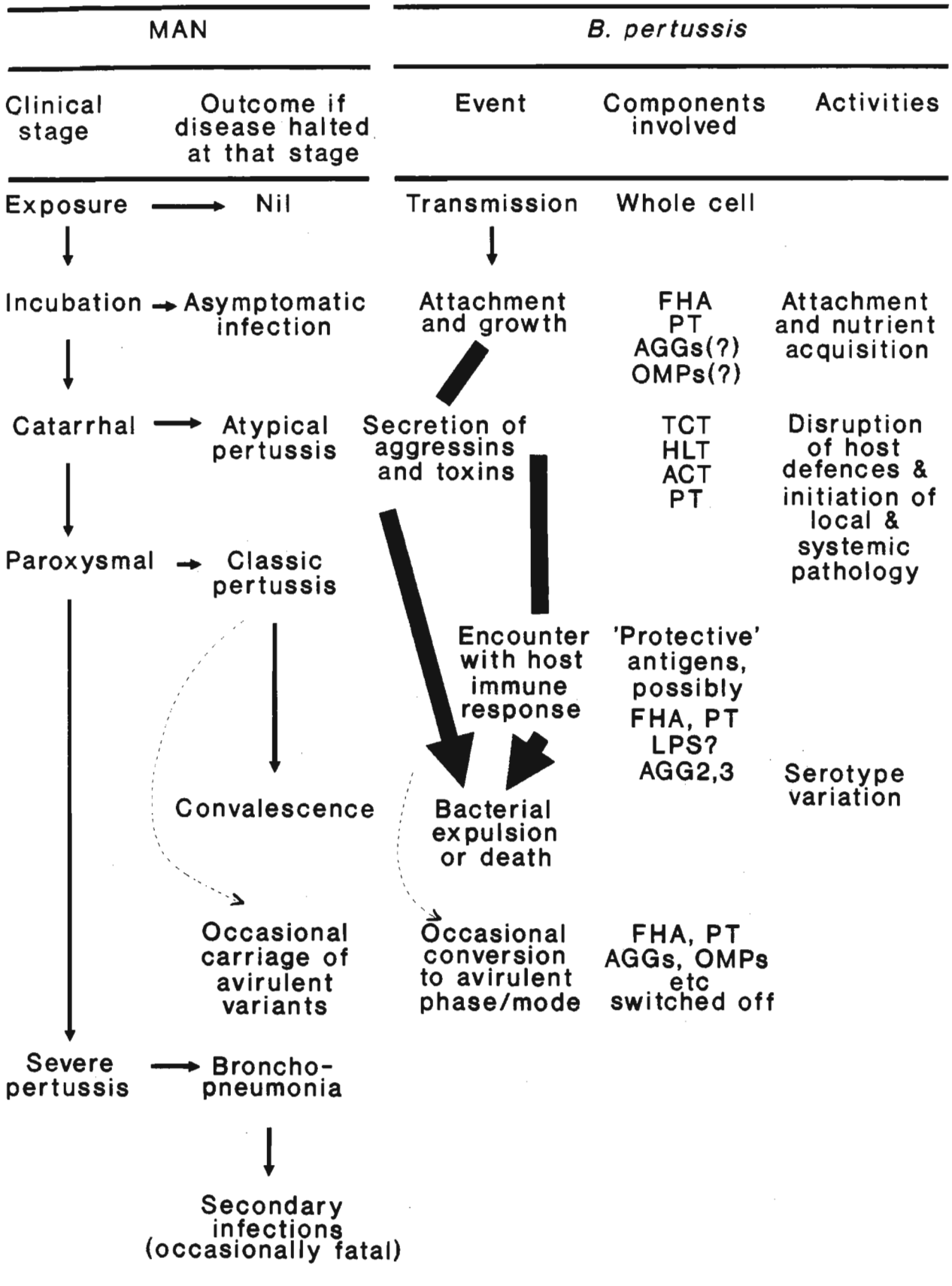

Figure 1.1 Diagrammatic representation of the possible sequence of events in pertussis (after Wardlaw & Parton, 1988).

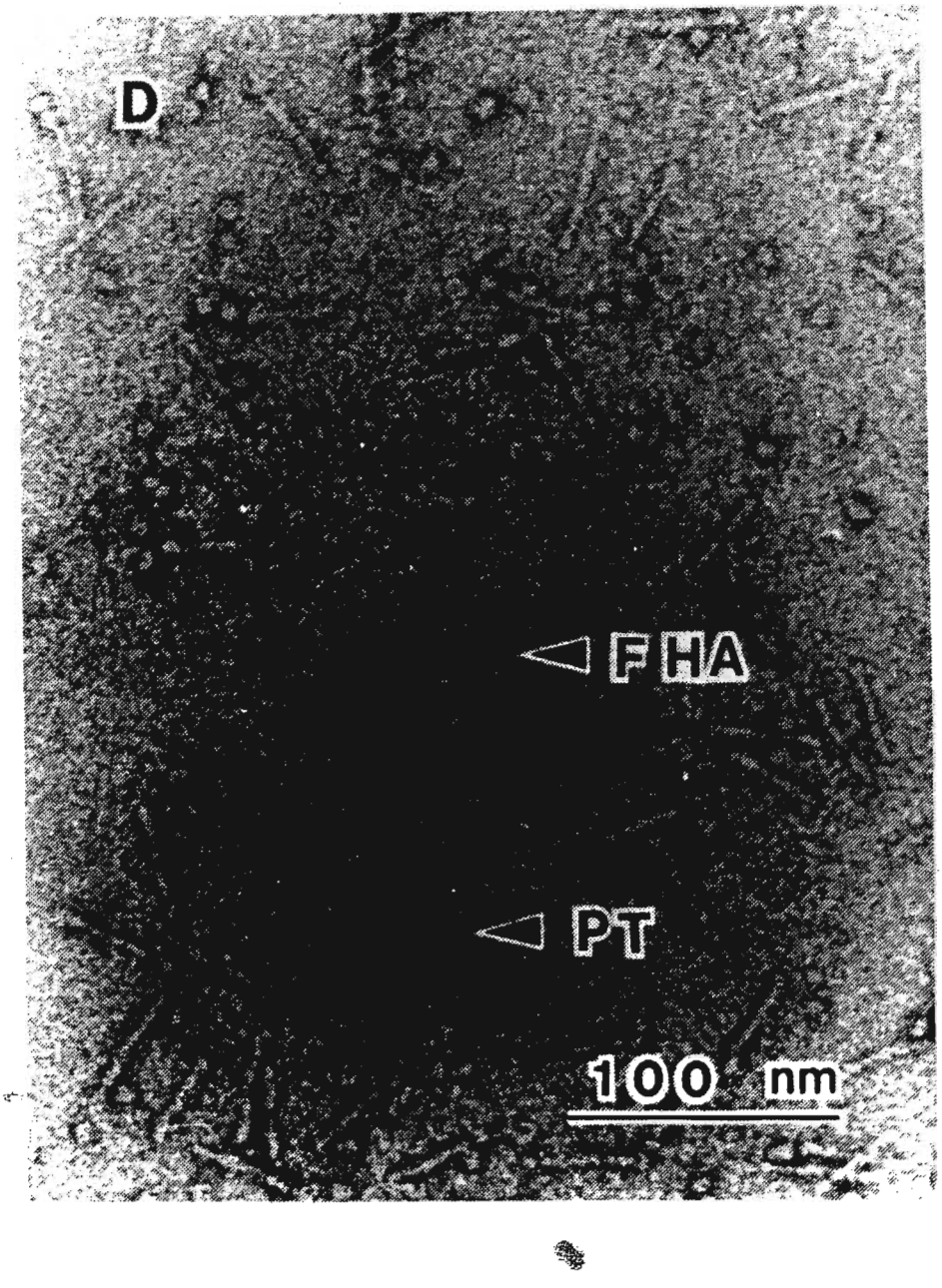

Figure 1.2 Electron micrograph showing pertussis toxin (PT) and filamentous haemagglutin (FHA).

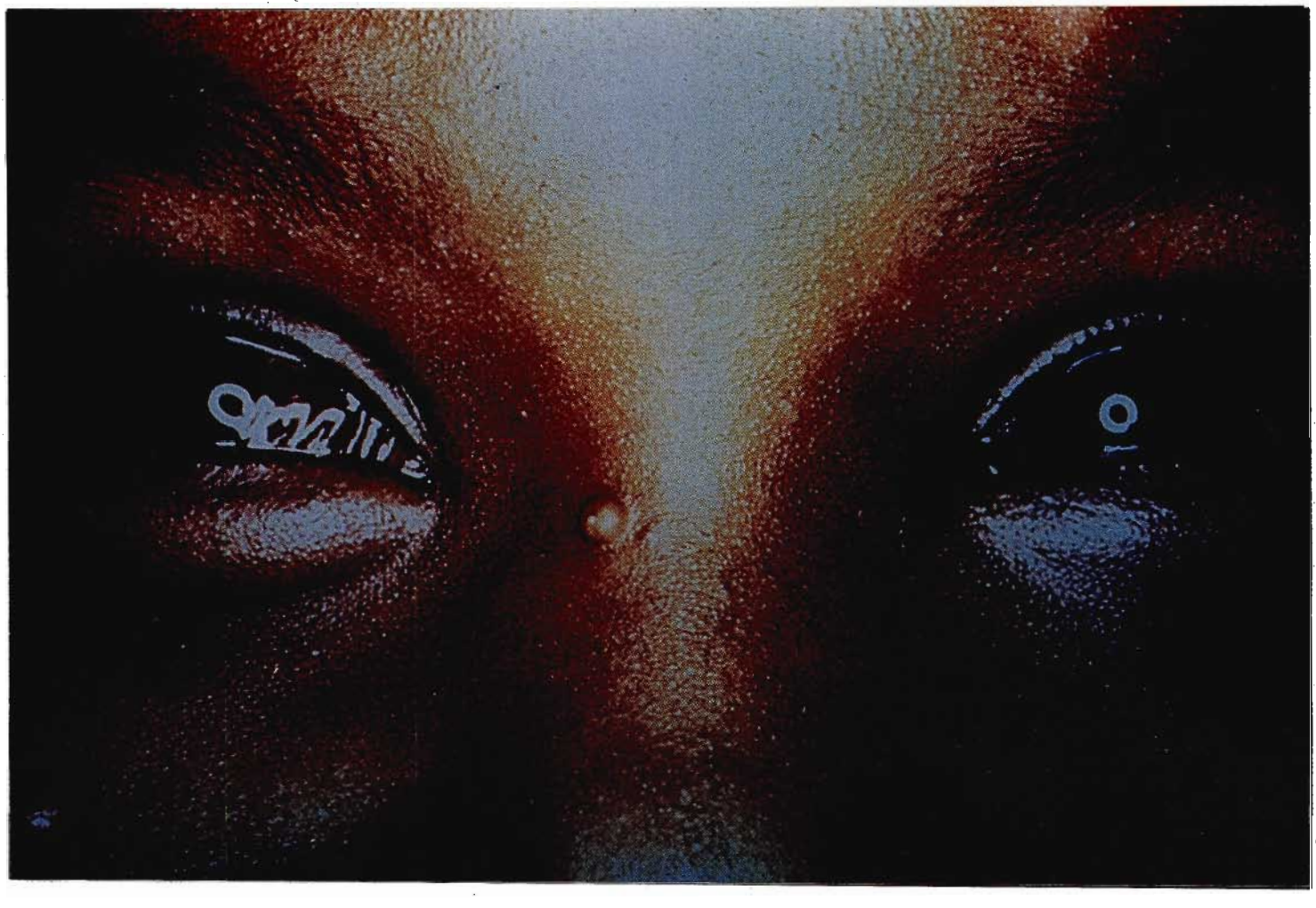

Figure 1.3 Subconjunctival haemorrhage during subsiding pertussis (Ajjan, 1989).

CHAPTER 2

VACCINATION AGAINST WHOOPING COUGH

2.1 INTRODUCTION

Whole-cell pertussis vaccines are currently in routine use throughout the world, except in Japan, Sweden and Italy (WHO, 1988) They have been given to over 1 billion children around the world during the past 30 years and are incorporated into the WHO Expanded Programme on Immunization (EPI). The vaccine consists of whole *B. pertussis* cells (P) that have been killed by heat or formalin treatment; and is usually administered in conjunction with diphtheria (D) and tetanus (T) toxoids as a combined product (DTP). Recommendations and schedules for vaccination vary significantly between countries.

Use of whole-cell vaccines is associated with a number of local and systemic adverse reactions ranging from mild to severe. In the 1970's vaccine uptake dropped in many countries as a result of public and governmental concern following observation of an unacceptably high incidence of severe, albeit uncommon, adverse reactions, including encephalopathy, neurologic damage and death (Ström, 1970; Stewart, 1977). There is much contention as to whether a direct causal relationship exists. Epidemiologic data do not support a statistical link, but it must re recognised that the whole-cell vaccine is among the crudest preparations routinely injected into humans. There is a possibility that the vaccine might trigger the earlier development of symptoms that would have occurred nevertheless. Several studies confirm that whole-cell DTP is associated with a greater frequency of acute neurologic illness than would be expected by chance, although the majority of affected children appear to recover completely.

Numerous studies of conventional whole-cell vaccine have arrived at considerable variations in efficacy estimates ranging from nil in Sweden during the late 1970's to approximately 95% when measured with reference to hospitalized cases in the United Kingdom (Fine & Clarkson, 1987). Conventional pertussis vaccines appear to raise titres of IgG but not those of IgA; to protect against disease to a greater extent than against infection; to protect against low but not high levels of challenge and to decrease in protective efficacy with time. The vaccine has been effective in reducing morbidity and mortality but not in decreasing the circulation of the

organism in countries where vaccination is widespread and although associated with side effects, its safety-benefit ratio is high when vaccine sequelae are compared to the morbidity and mortality caused by natural disease.

In recent years the identification and purification of several immunogenic antigens of *B. pertussis* whose antigenic and protective potential has been studied in animals and in humans, has led to the development of acellular pertussis vaccines devoid of bacterial cells. The development of an alternative pertussis vaccines arose from a need for better and more consistent protective efficacy against both infection and disease; the desirability of reducing the number of doses needed for effective vaccination; the desirability of removing toxic components, and thereby reducing any local and general reactions associated with such components, and the need to improve vaccine acceptance rates and restore public confidence after concern over the safety of whole-cell vaccines.

The term 'acellular pertussis vaccine' embraces vaccines differing widely in purity, characterization and sophistication, ranging from simple cell extracts or preparations from culture supernates; to defined acellular vaccines consisting of one or more components of known antigenic content and constituency; and subunit or synthetic peptide vaccines.

The first component pertussis vaccine was used in Japan in 1981 by Sato and co-workers and contained mainly PT and FHA. The vaccine is routinely given to children aged over two years in Japan and approximately 40 million doses have been administered to date. A number of acellular vaccines have since been produced by various methods and clinical trials are underway in the USA and other countries.

Acellular vaccines have shown lower toxicity than whole-cell vaccines and good efficacy and low reactogenicity in a number of clinical studies in developed countries. Available data indicates that although the number of local reactions seems unduly high, fever and other

systemic reactions will be less common than with whole-cell vaccines. Definitive data on the use of acellular vaccines in infancy is still awaited. Their effectiveness in reducing the incidence of whooping cough has been established in Japan in children 2 years of age or older. A series of collaborative studies, co-ordinated by WHO, are currently being undertaken in Sweden to evaluate the various preparations.

A number of issues pertaining to acellular vaccines remain unresolved, viz.

a. the optimum antigenic composition and dosage schedule.

b. whether antibody levels are predictive of protection and the determination of immunological correlates of protection against both the disease and carrier state.

c. the efficacy and long-term safety of acellular vaccines in infancy.

Until more is known about the relative roles played by the *b. pertussis* virulence factors, it will be difficult to decide which components to include in a "purified" vaccine. It seems likely that before an optimal vaccine composition can be decided, several large-scale field trials of vaccines comprised of various components in different ratios will have to be undertaken.

2.2 WHOLE-CELL VACCINES

2.2.1 DEVELOPMENT OF WHOLE-CELL VACCINES

Attempts to develop a vaccine against whooping cough started soon after *B. pertussis* was isolated and identified by Bordet & Gengou in 1906. The first pertussis vaccine to be used on a large scale was developed and tested by Madsen in Denmark in the 1920's. The vaccine consisted of freshly isolated organisms and some degree of protection was demonstrated in vaccine recipients during an epidemic in the Faroe Islands (Madsen, 1925,1933).

During the 1930's and 1940's improvements in growing *B. pertussis* and in killing, detoxifying

and preserving the organism, resulted in many different vaccine formulations, several of which proved quite effective. Evaluation of these early vaccines was based on results in clinical trials since no laboratory tests were available for measuring vaccine toxicity and potency (Sauer, 1933,1937; Kendrick & Eldering, 1936,1939).

During the middle 1940's convincing protective effects were demonstrated with pertussis vaccine combined with diphtheria and tetanus toxoids and adsorbed with aluminium hydroxide, aluminium phosphate or alum (Bell, 1941; Kendrick, 1942,1943). In 1944 pertussis vaccine was accepted and endorsed by the Council on Pharmacy and Chemistry of the American Medical Association.

The mouse intracerebral challenge protection test (ICMPT) was developed by Kendrick *et al.* in 1947 as a measure of vaccine potency and has continued to serve as a standard to assess pertussis vaccines. Extensive Medical Research Council (MRC) trials from 1946 to 1956 in the United Kingdom demonstrated a clear correlation between protection in mice and protection in children with this test (Medical Research Council, 1956).

Many attempts were made to develop improved pertussis vaccines that contained only the products necessary for vaccination in the 1940's and 1950's. In 1947 Pillemer *et al.* prepared an extracted vaccine that was protective in both humans and mice. The vaccine was however abandoned because of its reactogenicity. During the period 1960 to 1976 an extracted DTP vaccine (TriSolgen) containing a partially purified fractionated pertussis component was marketed in the United States by Eli Lilly Company. Product variability and no published evidence of its effectiveness however led to its discontinuation (Cherry *et al.*, 1988).

In 1964 an international standard pertussis vaccine was established based on the mouse ICMPT and it was recommended that vaccines be manufactured in accordance with WHO requirements (WHO, 1964).

Current regulations governing vaccine preparation were revised in 1979 by the Expert Committee on Biological Standardization of the WHO (1979). An international reference vaccine was established in 1980. This is kept at the Statens Serum Institut in Copenhagen and is used to standardize national preparations.

Adsorbed whole-cell DTP vaccine is now used in most countries. The number of killed cells in the vaccine (usually between $10x10^9$ and $16x10^9$ per dose), production strains, methods of manufacture and quality control procedures vary from country to country resulting in vaccines of different potencies.

2.2.2 MANUFACTURE OF WHOLE-CELL VACCINE

Whole-cell pertussis vaccines currently in use throughout the world are produced from a variety of different phase I strains of *B. pertussis*. The bacteria is cultivated either on solid media or submerged in liquid culture (Cohen-Wheeler medium) in a fermenter at 37°C for 2 days with shaking. The latter method allows considerable opportunity for antigenic variation. Cells are collected by centrifugation and washed with phosphate-buffered saline (PBS); then killed and detoxified either by heat treatment or chemically with formalin or thiomersal, or by long-term storage at 2-8°C, or a combination of these methods.

Pertussis vaccine may be distributed as a plain bacterial suspension or combined with diphtheria and tetanus toxoids and adsorbed onto an aluminium or calcium adjuvant which enhances the immune response (Preston, 1981) and reduces adverse reactions (Pollock *et al.*, 1984).

Vaccine potency is measured by the ICMPT and freedom from excessive toxicity is usually determined by the mouse weight gain test. In order to ensure the safety and efficacy of the final product the culture of each component strain of *B. pertussis* is tested for free formaldehyde

content (if formaldehyde is used to kill the bacteria); identity, toxicity, opacity, osmolality, purity, serologic composition and sterility before it can be accepted for combination with other strains or with diphtheria and tetanus toxoids. Figure 2.1 shows the manufacture of pertussis vaccine according to WHO (1979) specifications.

2.2.3 VACCINE USAGE

Age,dose and schedule: Since pertussis risk is highest in early infancy, it is preferable to start vaccination as early in life as possible. The recommended WHO EPI primary course consists of three doses commencing at 6 weeks of age with intervals of at least 4 weeks between doses, followed by booster doses at 15 to 18 months of age and again prior to school entry at 4 to 6 years of age. Vaccination schedules vary from country to country. The vaccine is not recommended after the seventh birthday because reactions are alleged to be increased significantly in older individuals and because the incidence rate and severity of pertussis infection decreases with age. The vaccine is given by intramuscular injection.

Several studies have investigated pertussis incidence after 2 and 3 dose schedules administered at various time intervals (Muller *et al.*, 1984; Bhandori, 1981; Bell, 1948). The available literature suggests greater protection following a 3-dose schedule, however in areas where access to health services is limited, a 2-dose schedule may be considered in order to reduce the number of required contacts with the health services. Wilkins *et al.* (1971) suggested that the administration of 2 DTP vaccinations spaced >60 days apart was adequate.

Pertussis vaccination commenced within 24 hours of birth followed by 2 doses at monthly intervals did not produce an adequate serologic response (Dengrove *et al.*, 1986). Butler (1962) reported adequate protection when the primary series was given at 1, 6 and 12 weeks, followed by a booster at 1 year of age.

Contraindications: These vary in different countries. In the United Kingdom a family history of

neurological disease and developmental defects are contraindications to DTP vaccination but are not in the United States. Minor illnesses such as mild respiratory infections, diarrhoea, low-grade fever are not contraindications to vaccination, however any severe febrile illness, particularly respiratory, is a reason to defer vaccination (Galazka *et al.*, 1984).

Vaccination should not be repeated in children who have a history of any severe local or general reaction, or a neurological reaction to a preceding dose. These include fever $\geq 40.5^{\circ}C$ within 48 hours, seizures with or without accompanying fever, collapse or shock-like states, persistent screaming episodes, encephalopathy, systemic allergic reactions (urticaria, bronchospasm, laryngeal oedema), extensive erythema and swelling. Vaccination is contraindicated in children with a history of cerebral irritation or damage in the neonatal period, or who have suffered from fits or convulsions.

Special consideration should be given to the advisability of vaccinating children in the following groups in which the vaccine is not absolutely contraindicated (the risk of vaccinating may be higher, but the effects of whooping cough may also be more severe and the benefits of vaccination therefore greater).

a. children whose parents or siblings have a history of idiopathic epilepsy.

b. children with developmental delay thought to be caused by a neurological defect.

c. stable neurological conditions.

SIDS or severe reaction following administration of pertussis vaccine in a family member is not considered a contraindication to vaccination.

Some studies suggest that vaccination be delayed in babies with a high risk of allergy or atopy to prevent atopic sensitization (Haus 1987,1988), however allergy is not a contraindication to pertussis vaccine. Malnutrition is considered an indication rather than a contraindication.

There is no evidence that the response to whole-cell DTP vaccine is inadequate in malnourished children (Muller *et al.*, 1987).

2.2.4 ADVERSE REACTIONS

It is well recognised that whole cell pertussis vaccine is the most reactogenic of the commonly used vaccines for routine vaccinations (Pollock *et al.*, 1984; Long *et al.*, 1990a). Infants who experience a reaction after one dose have an increased tendency to react adversely to a subsequent dose. Approximate rates for adverse events following receipt of DTP vaccine (regardless of dose number in the series) are indicated in Table 2.1 (Cody *et al.*, 1981; Barkin & Pichichero, 1979; Pollock *et al.*, 1984; Miller *et al.*, 1981; Manclark *et al.*, 1984; Recommendations of the Immunization Practice Advisory Committee, 1985).

LOCAL REACTIONS

The occurrence of mild local reactions (pain, erythema, heat, oedema, induration with or without tenderness) at the injection site are common within 48 hours following vaccination and develop in 25 to 50% of vaccinees (Barkin & Pichichero, 1979; Cody *et al.*, 1981). Occasionally, a nodule may be palpable at the injection site of adsorbed products for several weeks. Sterile abscesses have also been reported (6-10 per million doses). Local reactions, range in size from 3 to >7.5 cm, depending upon the number of doses received, and have been reported to occur more frequently after the third dose (Pollock *et al.*, 1984).

SYSTEMIC REACTIONS

Mild systemic reactions: Occur in 10 to 25% of vaccinees within 12-24 hours following vaccination and include crying, screaming, low grade fever, drowsiness, fretfulness, vomiting and anorexia (Barkin & Pichichero, 1979; Cody *et al.*, 1981; Pollock *et al.*, 1984). A significantly higher rate of mild reactions following DTP than following DT vaccination was demonstrated by Cody *et al.*, 1981. The reactions are usually self-limited and need no therapy other than perhaps, symptomatic treatment, eg. antipyretics. Rash, allergic reactions, and respiratory

difficulties including apnoea have been observed. Other than fever, the frequency of mild to moderate systemic reactions is significantly less frequent with increasing numbers of doses of DTP (Cody *et al.*, 1981).

Moderate systemic reactions: These occur relatively infrequently, (approximately 2.5 per 1000) injections and are characterised by fever $\geq 40.5^{\circ}C$, unusual and prolonged crying (lasting 3 hours or more), abnormal high-pitched screaming, twitching and jerking, pallor and cyanosis (Pollock *et al*, 1984; Long *et al.*, 1990a).

SERIOUS SYSTEMIC AND NEUROLOGICAL REACTIONS

Although infrequently observed, a significant association has been shown between serious neurological illness and pertussis vaccine (Miller *et al.*, 1981). Most are unlikely to result in lasting sequelae. These include simple febrile convulsions of less than 10 minutes duration occurring within 24 hours post-vaccination in a child who is otherwise developmentally and neurologically normal prolonged convulsions, and encephalopathy, occasionally fatal. These occur in 1:1,750 to 1:13,400 vaccinations. Anaphylaxis (eg. hives, swelling of the mouth, difficulty in breathing, hypotension, or shock) may occur within two hours after vaccine administration (Cody *et al.*, 1981; Pollock & Morris, 1983; Walker *et al.*, 1988).

Epidemiologic studies have failed to document a direct causal relationship between the administration of whole-cell pertussis vaccine and the onset of serious acute neurologic disorders followed by permanent sequelae (Walker *et al.*, 1988; Cody *et al.*, 1981; Shields *et al.*, 1988). The risk of serious neurologic reactions following administration of whole-cell pertussis vaccine is much lower than the risk from natural pertussis disease. These are shown in Table 2.2.

It has been suggested that the vaccine may trigger the onset of neurologic symptoms in those children who were destined to develop symptoms (Bellman *et al.*, 1983). There is no evidence

that vaccine-associated convulsions produce central nervous system (CNS) injury, worsen pre-existing neurologic disease, or herald the onset of epilepsy (Shields *et al.*, 1988; Baraff *et al.*, 1986).

1. Convulsions (complex febrile/afebrile). Various authors have estimated the frequency of convulsions following pertussis vaccination to be no higher than that observed in an unvaccinated population of the same age. WHO evaluates the background monthly incidence rate of convulsions to be 0.8 to 1.4 per 1000 children aged 6 to 18 months. The incidence of post-vaccination convulsions is relatively common and is estimated at about 1 case per 10,000 doses, with variations from one study to another, as shown in Table 2.3 (Ajjan, 1989). In most cases recovery occurs without sequelae. Available data suggest that infants and young children who have had previous convulsions (febrile or non-febrile) are more likely to have seizures following DTP than those without such histories (Recommendations of Immunization Practices Advisory Committee). Onset of infantile spasms has occurred in infants following DTP or DT. Several controlled studies have shown no causal relationship between infantile spasms and whole-cell vaccination (Shields *et al.*, 1988; Fukuyama *et al.*, 1977; Melchior, 1977). The incidence of infantile spasm increases at 3-9 months of age, the time period in which the second and third doses of DTP are given. Therefore some cases can be expected to occur by chance alone.

2. States of shock/collapse. These are shock-like episodes with signs of vascular collapse, muscle hypotonicity and unresponsiveness for up to 10 minutes or more, with or without fever, but without paralysis or seizures and occurring 6-10 hours post-vaccination. Onset is sudden, and is accompanied by pallor, occasional cyanosis and agitation. No adverse long-term effects have been reported. Personal and familial history of allergy are noted in about half of these cases. The frequency of collapse/shock has been estimated at about 0.1-1% (Cherry *et al.*, 1988) and varies in several studies. These are shown in Table 2.4

3. Encephalopathies. Encephalopathies generally occur within 24 hours of vaccination and seldom after 72 hours. Signs include apathy, restlessness, poor appetite and shrill crying. Epidemiological evidence does not support an association between encephalopathy and administration of whole-cell vaccine in view of the absence of specific clinical features and consistent neuropathological findings.

The incidence of post-pertussis vaccination encephalopathies occurring in the 14 days following whole-cell DTP vaccination has been estimated to be between 1:25,000 to 1:60,000 vaccinations (Ross, 1989) and the estimated attributable risk to be 1/110,000 doses (Miller *et al.*, 1981,1985); whether this represents an increase in the background rate is not established. The WHO estimates the frequency of encephalopathy (including epileptic seizures, local neurological signs, comas and Reye's Syndrome) to be from 0.09 to 4%. Other investigators have estimated the occurrence of pertussis vaccine encephalopathy to be between 1 case/54,000 children and 1 case/400,000 children in uncontrolled studies (Cherry *et al.*, 1984).

4. Fatal complications. Sporadic reports of sudden infant deaths following pertussis vaccination have occurred (Baraff *et al.*, 1983; Bernier *et al.*, 1982). Death usually occurs after a long series of severe neurological complications. The exact incidence and causal factor remain unknown. All controlled studies have however shown no causal relationship between Sudden Infant Death Syndrome (SIDS) and whole-cell vaccination (Hoffman *et al.*, 1987; Griffin, 1988; Walker *et al.*, 1987).

5. Hypsarrhythmias. Some rare cases have been attributed to pertussis vaccination, however no formal proof of a causal relationship between the two exists. Bellman *et al.* (1982) hypothesizes the existence of a specific disposition facilitating the occurrence of hypsarrhythmias, the vaccine then triggers the reaction in children who would have developed the condition in any event.

6. Others. The probability of any of the following clinical conditions being causally-related to whole-cell pertussis vaccination was considered to be nil: serum sickness, aseptic meningitis, Reye's Syndrome, peripheral mononeuropathy, transverse myelitis, chronic brain syndrome in the absence of acute encephalopathy (Bellman *et al.*, 1982). No evidence of additional brain damage following pertussis vaccination in children with tuberous sclerosis has been reported (Hunt, 1983).

2.2.5. PATHOGENESIS OF VACCINE REACTIONS

Whole-cell pertussis vaccine may contain up to 3,000 different antigens, some of which are necessary for protection, most are useless and many are toxic and are probably responsible for the observed side effects. There is a lack of specific information relating to the cause and mechanism of vaccine reactions.

Several antigens of *B. pertussis* are known to have toxic properties, viz. PT, ACT, TCT, LPS endotoxin. Most speculation has centred on the possible roles of PT and LPS endotoxin in causing serious events. Whole-cell vaccines contain high concentrations of LPS endotoxin, a known cause of fever, inflammation and shock. Recent trials in the United States (Edwards *et al.*, 1986; Pichichero *et al.*, 1987) found that fever, local and common systemic symptoms occurred more commonly following whole-cell vaccine than following an acellular vaccine containing 95% less endotoxin. It is probable that many uncomplicated seizures following pertussis vaccination are due to fever caused by endotoxin and are dependent on host susceptibility factors. There was a slight positive relationship between pain at the injection site, fever and endotoxin content (Baraff *et al.*, 1986) Endotoxin does not play an important role in protection from pertussis and may therefore be eliminated from acellular vaccines. The causes of persistent crying and hypotonic-hyporesponsive episodes are not known.

In Sweden, Hedenskog *et al.*, (1987) found a higher incidence of local reactions following vaccination with a preparation containing PT and FHA, than after one containing PT alone,

suggesting that FHA and perhaps other surface antigens may be in part responsible for local reactions. Murphy *et al.* (1983) reported an association between increased incidence and severity of local reactions when aluminium phosphate was used as an adjuvant in whole-cell vaccine.

The pathogenesis of pertussis encephalopathy remains unclear and the lack of an animal model has retarded understanding. Pittman (1979,1984) has suggested that PT is involved in the pathogenesis of CNS reactions. Detoxified whole-cell vaccines are known to contain histamine sensitizing-factor (HSF) and active PT (Ashworth *et al.*, 1983) which is capable of producing lymphocytosis in vaccinated children and has caused death by anaphylaxis in animals (Steinman *et al.*, 1985; Munoz *et al.*, 1987). Following studies on the effect of pertussis vaccine on cerebral vascular permeability in mice, Amiel (1976) has suggested that increases in permeability in the cerebral vessels (a direct effect of PT) may be involved in the evolution of encephalopathy.

PT has been shown to cause altered insulin-glucose homeostasis, and hence hypoglycaemia (Yajima *et al.*, 1978; Sekura *et al.*, 1985). Whole-cell pertussis vaccination of mice produces increased insulin secretion, hypoglycaemia and impairment of adrenalin-induced hyperglycaemia (Gulbenkian *et al.*, 1968; Sumi & Ui, 1975; Pittman, 1980; Furman *et al.*, 1986; Hewlett *et al.*, 1983). Von Hennessen & Quast (1979) pointed out the close symptomatic similarities between hypoglycaemia and severe reactions to pertussis vaccine in an analysis of 149 vaccinated infants with reported reactions to whole-cell vaccine, of whom 59 had shown severe reactions and 13 had died. No blood glucose data were available. Clinical symptoms of hypoglycaemia may have been overlooked since infants may not present with classic neurogenic symptoms and convulsions may be the only sign (Marks & Clifford, 1981). Infants who are genetically predisposed to hypoglycaemia and hyperinsulinaemia when exposed to PT, may develop inappropriately high insulin levels with resultant hypoglycaemia. The possible contribution of hypoglycaemia to the serious reactions attributable to pertussis vaccine must

be considered in these infants. No studies have however documented clinically significant post-vaccination hypoglycaemia in children.

2.2.6 EFFICACY OF WHOLE-CELL VACCINE: EARLY STUDIES

The efficacy of pertussis vaccine is conventionally defined as the percentage reduction in disease risk in vaccinated individuals as compared to equally exposed unvaccinated individuals. In the numerous clinical trials of pertussis vaccine that have been performed since the mid-1920's, protective efficacy has varied considerably. Early studies in the United States in the 1930's and early 1940's with plain whole-cell pertussis vaccines demonstrated some protection in children (Madsen, 1925,1933; Sauer 1937). However, protection trials in the United Kingdom at this time produced disappointing results; no significant protection from disease was noted (McFarlan *et al.*, 1945). In view of these conflicting results the Medical Research Council of the United Kingdom organised a series of controlled trials in the 1950's using the vaccines of 5 different manufacturers in order to assess protective activity and to determine whether this activity could be correlated with laboratory tests. Results showed that the vaccines varied considerably in protective efficacy, but nevertheless, demonstrated an efficacy of up to 95% in preventing secondary cases in household contacts (MRC 1951,1956,1959). Routine vaccination against pertussis was introduced in most industrialized countries in the late 1950's or early 1960's.

Between 1950 and 1962 the incidence of pertussis declined greatly in the United Kingdom, however between 1963-64 an epidemic occurred. Incidence of pertussis between 1940 -1982 is shown in Figure 2.2. A survey by the Public Health Laboratory Services (PHLS) found that some of the vaccines in use were well below the potency required internationally, and that the serotypes of the prevalent *B. pertussis* strains were not incorporated in the vaccine.

The contribution made by pertussis vaccination to the decline in incidence and severity of the disease has been vigorously debated. In both the United Kingdom and the United States,

deaths from pertussis had already fallen before the widespread use of whole-cell vaccine This was thought by some to be a consequence of improved social standards.

Since the Medical Research Council trials in the 1950's there have been no further double-blind field trials of whole-cell pertussis vaccines. More recent assessments of vaccine-efficacy have been by indirect methods (observations of disease severity and vaccination status of cases, and by examination of attack rates in the community).

2.2.7 EFFICACY OF WHOLE-CELL VACCINES: RECENT STUDIES

Vaccine efficacy is conventionally defined in terms of protection against clinical disease. There is evidence that whole-cell vaccines may protect against infection to a lesser extent than they do against disease (PHLS, 1982).

Fine & Clarkson (1987) summarized efficacy data for whole-cell pertussis vaccines derived from nearly 50 trials worldwide (Table 2.5). Efficacy rates varied from nil to 95%, depending on case-definitions, age groups and vaccines used. The wide range of observed differences in vaccine efficacy may be in part attributable to the following factors -

i. Variations in pertussis vaccination schedules between countries and over time.
ii. Differences between the antigenic composition of the vaccine used and that of the circulating strain of *B. pertussis*.
iii. Differences in the composition and preparation of various vaccines.
iv. Difficulties in diagnosing pertussis.

Several studies of vaccine efficacy against reliably diagnosed pertussis (Noah, 1976; Preston, 1976b; Church, 1979; McKendrick *et al.*, 1980) support the view that current whole-cell vaccines are effective in protecting the individual against typical whooping cough, either by preventing its occurrence altogether or by markedly reducing its severity.

Routine mass vaccination in both the United States and the United Kingdom has led to the successful control of pertussis. Prior to the use of pertussis vaccine 115,000-270,000 cases of pertussis and 5,000-10,000 deaths due to the disease occurred yearly in the United States. During the last 10 years this has fallen to 1,200-4,000 cases and 5-10 deaths per annum (Centers for Disease Control, 1984). Epidemiological studies show that the vaccine played an important role in these reductions (Fulginiti, 1984).

The protective efficacy of whole-cell vaccines has been questioned because of high attack rates reported in vaccinated individuals in several studies (Bröms & Ljung, 1980; Miller & Fletcher, 1976; Stewart, 1977,1981; Ditchburn, 1979; Grob *et al.*, 1981; Long *et al.*, 1990b). Whole-cell vaccine was shown to be 80-90% effective in preventing disease in fully-vaccinated children who were exposed to household contacts (Broome & Fraser, 1981).

2.2.8 CONTROVERSY

High rates of immediate adverse reactions (temperature elevation, local reactions, behavioural changes) as well as some neurological complications (encephalopathy, infantile spasms complex febrile and afebrile convulsions; with long-lasting sequelae and death as possible consequences have been described following whole-cell pertussis vaccination (Fulginiti, 1984; Hinman & Kaplan, 1984). Since 1933 there has been prolonged debate concerning the existence of a causal relationship between neurological symptoms and pertussis vaccination. There has also been continuing controversy about whether the benefits of routine vaccination for pertussis outweigh the potential risks of infection.

The risk of the more uncommon but severe complications have been emphasized by the media and have caused much concern in the lay and medical population. However, in the absence of epidemiologic or specific pathologic evidence of a cause and effect relationship, there has been much contention as to whether the vaccine does cause serious reactions.

Several studies were performed to examine the risk of acute neurological illnesses after whole-cell pertussis vaccination. In three large case-controlled studies which involved about 230,000 children and 713,000 doses of vaccine, no evidence of a causal relationship between pertussis vaccine and permanent neurological damage was found. Only an association between vaccination and first febrile seizures was established (Griffith *et al.*, 1982; Walker *et al.*, 1988; Shields *et al.*, 1988). This was explained by the presence of native pertussis endotoxin in whole-cell vaccines.

In a vast case-controlled investigation in Britain, the National Childhood Encephalopathy Study (NCES) (Miller *et al.*, 1982), showed the estimable attributable risk of neurological problems within 7 days of vaccination to be 1 in 110,000 and of permanent neurological sequelae to be or 1 in 310,000 injections. Further follow-up and reanalysis of this study suggests that these frequently quoted incidence figures are too high (Dyer, 1988; Miller *et al.*, 1981,1982) and that the data do not provide conclusive evidence that the vaccine is responsible for such side effects. In addition, if all cases of Reye's Syndrome and proven viral disease were excluded from the study cases, no statistical evidence of permanent brain damage and no increase in deaths could be related to the vaccine. Hence no consistent spectrum of pertussis-vaccine associated neurological illness emerged. The results of the NCES and other studies did however confirm that whole-cell pertussis vaccine was associated with a greater frequency of neurologic illnesses than would be expected by chance, although the majority of affected children appear to recover completely (Miller *et al.*, 1982).

The vaccine is administered at the very age when children are most likely to present with the first signs of underlying neurological disease which may appear coincidentally at DTP administration or are brought out by the common systemic effects of DTP vaccination or any other fever-producing event. In addition, available data indicate that the onset of inevitable neurological illness may be moved forward in time by vaccination (Bellman *et al.*, 1983).

Neurologic events following vaccination appear to be chance temporal associations of neurologic conditions that occur in the target age group even in the absence of vaccination (Golden, 1990). The 'background rate' or expected prevalence of a first seizure in any of the 5 days following any notable event in a healthy child aged 3-6 months is 1 in 15,000 (Turnstall-Pedoe & Rose, 1981). In the United Kingdom, serious encephalitic,or acute neurologic disease (not due to bacterial infection, trauma or poisons) affects approximately 1 in 2,000 in the first year of life (Miller *et al.*, 1981).

Sporadic case reports of children who incurred neurological disorders following pertussis vaccine have been published in the past 50 years (Byers & Moll, 1948; Berg, 1958; Kulenkampff *et al.*, 1974; Cavanagh *et al.*, 1981). None of the series included controls or attempts to place these problems in the general context of childhood neurological illness. Recent literature does not support the existence of pertussis vaccine encephalopathy and no causal relationship between the two has been proven.

Since 1978, several investigators have produced risk-benefit analyses of pertussis vaccination (Koplan *et al.*, 1979; Mortimer & Jones, 1979b; Hinman & Koplan, 1984). When compared with the prolonged routine morbidity of pertussis and the frequency of death or permanent sequelae, the risk associated with vaccination has no relevance. This is especially true in developing countries where incidence and prevalence of pertussis is high. In such countries, the value of increasing vaccination coverage is supported by the WHO through its Expanded Programme on Immunisation.

Official policy in most industrialized countries continues to recommend routine pertussis vaccination of all infants since the weight of scientific evidence still favours this practice. Nevertheless, in countries where the use of whole-cell vaccines is widespread and where both pertussis incidence and mortality has declined, the question arises as to whether continued

vaccine usage now constitutes a greater risk than the disease itself (Stewart, 1977).

2.2.9 EFFECT OF CONTROVERSY IN INDUSTRIALIZED COUNTRIES

Governmental and public concern about possible pertussis vaccination reactions and efficacy resulted either in a marked decline in vaccine acceptance or official discontinuation of routine pertussis vaccination in the United Kingdom, Japan, Italy, Sweden and parts of West Germany in the early 1970's following adverse media publicity.

Public concern in the United Kingdom that pertussis vaccine might be associated with neurological problems followed publications by Kulenkampff *et al.* (1974) and by Aicardi & Chevrie (1975) wherein children who developed encephalopathic illness after pertussis vaccination were described. Neither of these series included controls or attempts to place these problems in the general context of childhood neurological illness.

Nevertheless, adverse media publicity led to a fall in the overall pertussis vaccination rate from 77% in 1974 to less than 30% in 1978 (Griffith, 1981) and subsequently the incidence of pertussis cases increased greatly, reaching 102,900 per year (Cherry *et al.*, 1988). Usage fell despite the fact that vaccine efficacy ($\widehat{VE}$) was calculated to be 80% for cases defined clinically and 93% for laboratory confirmed cases during this period. Two epidemics occurred, one in 1977-1979 (110,000 cases) and a second in 1982-1983 (Pollard, 1980; HMSO, 1981).

In Japan, routine whole-cell pertussis vaccination was suspended in 1975 following 2 infant deaths (1 from encephalopathy and 1 from shock) within 24 hours of vaccination (Kimura, 1986; Noble *et al.*, 1987; Takayama, 1989). By 1976 the vaccination rate was 13.6% and a dramatic increase in pertussis incidence and mortality occurred, mostly in children under 2 years of age (Kanai, 1980; Sato *et al.*, 1984; Kimura & Hikino, 1985; Isomura, 1988). From 1975-1979 all children received DT instead of whole-cell DTP, with the initial dose being given at 2 years of age. A major epidemic of pertussis occurred in 1979 with over 13,000 cases

reported and an incidence rate >10 per 100,000 population. In 1980 whole-cell DTP was reintroduced at 2 years of age. This was replaced by acellular vaccine in mid-1981, also given at 2 years of age. Current estimations of vaccine coverage are approximately 70%.

In Sweden in the late 1970's public concern regarding possible neurologic vaccine reactions and insufficient protective efficacy resulted in lowered vaccine acceptance. Official discontinuation of pertussis vaccination occurred in 1979 (Cherry *et al.*, 1988; Romanus *et al.*, 1987). A rise in reported cases of pertussis resulted; between 1980 and 1985, >36,000 cases of pertussis were confirmed. Currently, routine vaccination is not administered and the annual incidence of disease is 4-5% in young children. As a result of the low uptake of vaccination there has been a higher proportion of older children notified in recent epidemics, reflecting a relative lack of herd immunity.

Pertussis incidence remains high in several European countries, including those which do not use pertussis vaccine routinely in their infant vaccination programmes (Sweden, Italy). The major epidemics which occurred in the United Kingdom, Sweden and Japan provide powerful evidence of the effectiveness of whole-cell vaccines, and reinforce the view that these vaccines confer useful, though not complete, protection.

2.3 ACELLULAR AND DEFINED PERTUSSIS VACCINES

2.3.1 POTENTIAL VACCINE ANTIGENS

Recent research on the host-parasite interactions in pertussis and in the immunochemistry of *B. pertussis* has identified several candidate immunogens for inclusion in acellular vaccines (Robinson *et al.*, 1985,1988; Weiss & Hewlett, 1986; Wardlaw & Parton, 1988) (Table 2.6).

An optimal vaccine against pertussis should contain antigens intended to elicit protection against attachment and growth stages, and against the pathogenic effects of the toxin, without

adverse reactions.

The inclusion of a particular antigen in a vaccine is dependent upon its ease of isolation in high yields, its role in pathogenesis (supported both theoretically and experimentally), the demonstration of its immunogenicity and protective potency in some animal models of pertussis, and evidence that it induces functional antibodies in children after effective vaccination with the whole-cell vaccine. There is nearly universal agreement that appropriately detoxified PT and FHA should be components of acellular vaccines, although recent Swedish studies failed to correlate antibodies with protection (*Ad hoc* Group for the Study of Pertussis Vaccines, 1988). Some authors believe that a new vaccine should also contain defined amounts of agglutinogens in addition to PT and FHA.

Among the candidate antigens for inclusion in defined component pertussis vaccines (Table 2.6) are the following -

Pertussis toxin: PT is considered to be the essential immunogen for immunising man against pertussis. The toxin is thought to play a major role in the pathogenesis of pertussis and it is also believed to be the major antigen. All acellular vaccines currently on trial (Table 2.6) contain toxoided PT, with or without FHA and other components (Griffiths & Kreeftenberg, 1985; Lewis *et al.*, 1986; Kallings & Olin, 1987). Some believe that PT is an essential adjuvant for making other pertussis antigens protective. FHA was found to have a synergistic action with PT in animal experiments (Pittman, 1984; Sato & Sato, 1984).

Several investigations with purified PT and its antibody in mice have identified it to play a major role in protection against pertussis infection (Sato & Sato, 1983; Oda *et al.*, 1984; Sato *et al.*, 1983). Immunisation of mice with PT protected them against both aerosol challenge (Cowell *et al.*, 1984) and intracerebral challenge (Sato *et al.*, 1981). Human antibodies to PT also passively protected suckling mice from aerosol infection with *B. pertussis* (Oda *et al.*, 1984).

Anti-PT antibodies are thought to play a role in long-term protection against the disease. However there are some case reports of convalescents who do not have detectable anti-PT antibodies in serum. The significance of cellular immunity to PT is not clarified.

Active PT must be chemically inactivated, to destroy its toxic activity whilst retaining its immunogenicity before incorporation into a vaccine. PT subjected to inactivation has been found to be partially reverted to active toxin in some acellular products. The toxicity of PT is assessed by haemagglutination, ADP ribosylation, CHO cell aggregation, lymphocytosis and histamine sensitization. Alternative approaches are the production of immunogenic non-toxic components of PT by genetic manipulation.

Filamentous haemagglutin: FHA is a principal candidate for inclusion in acellular pertussis vaccines because of its role in adhesion of *B. pertussis* to human respiratory cells during the initial colonization phase of infection. In addition, it is non-toxic, highly immunogenic, and easily isolated from culture supernates of *B. pertussis* in high yields (Imaizumi *et al.*, 1984).

Various studies have shown the active protective role of FHA against respiratory infection of rabbits (Ashworth *et al.*, 1982a; Irons & MacLennan, 1979) and the passive protection of FHA antibodies against aerosol infection of mice (Sato *et al.*, 1981a,b; Sato & Sato, 1984; Robinson *et al.*, 1985a; Oda *et al.*, 1983). However anti-FHA was found to be non protective against ic challenge in mice (Sato *et al.*, 1981).

Recent follow-up studies of unvaccinated Swedish children without a history of whooping cough but with antibodies to *B. pertussis* antigens indicated that anti-PT but not anti-FHA may be protective against the disease (Zackrisson *et al.*, 1990). Anti-FHA antibodies are thought to play a role in the short-term protection against pertussis (Winsnes *et al.*, 1985). Granström *et al.* (1982a) reported a rapid decrease in anti-FHA antibodies in long-term follow-up of culture-verified pertussis patients indicating that these antibodies are not important in long-term

protection. No correlation occurred between anti-FHA antibody levels and duration of symptoms during whooping cough (Viljanen *et al.*, 1985).

Agglutinogens: Three major antigens of *B. pertussis*, AGGs 1, 2 and 3 are considered to be important in the epidemiology of pertussis (Olson, 1975). Vaccines containing AGGs 1 and 2 were quite effective in Great Britain in the 1960's, but decreased efficacy was noted in the 1970's, presumably because of the emergence of type 1 and 3 organisms. The use of strains bearing type 1 and 3 agglutinogens in the vaccine correlated with improved efficacy. It is recommended by the WHO that strains with the serotype antigens 1, 2 and 3 be included in vaccines. The fimbrial nature of AGGs 2 and 3 strengthen their importance as potential vaccine components because of known involvement in adherence. However the protective role of antibodies to AGGs 2 and 3 remains unclarified. Neither AGGs nor agglutinins protected mice against ic challenge (Eldering *et al.*, 1966; Eldering *et al.*, 1967; Robinson *et al.*, 1985). They did however render some protection against intranasal infection (Robinson *et al.*, 1985; Zhang *et al.*, 1985). Isolated AGGs have been found to be protective provided they contained a trace of biologically active PT (Robinson & Irons, 1983; Robinson *et al.*, 1985).

In MRC vaccine trials in the United Kingdom in the 1950's a good correlation was found between the agglutinin response in mice and protection in children (MRC, 1959). A report (Askelöf, 1985) casts doubt on the importance of the AGGs by the finding that after the natural disease up to 50% of children had no measurable antibody titres against AGGs but were immune to the disease. Nevertheless, AGGs 2 and 3 have been purified and have been incorporated into experimental vaccines. Opinion about their importance remains divided despite both laboratory and epidemiological evidence (PHLS, 1973) suggesting that they are involved in protection.

69 Kilo Dalton (kDa) Outer Membrane Protein (OMP): The presence of a 69 kDa non-fimbrial OMP in all virulent strains of *B. pertussis* (Brennan *et al.*, 1988), and its ability to induce

agglutinating antibodies together with evidence of protective efficacy in mice following intra-nasal challenge (Shahin *et al.*, 1989) makes this protein a candidate for inclusion in component acellular vaccines.

Antibody responses to 69 kDa OMP have been demonstrated in children 3 to 9 months after whole-cell pertussis vaccination, and in serum and saliva after pertussis infection (Blumberg *et al.*, 1990; Thomas *et al.*, 1989). Also, T-cell clones from convalescents proliferated specifically in response to 69 kDa stimulation (De Magistris, 1988). Thus 69 kDa OMP elicits both B-cell and T-cell mediated immunity.

Recently significant amounts (2-3 mcg) of the protein have been found in the Japanese T-type (Takeda) acellular pertussis vaccine. A marked IgG-anti-69 kDa OMP response one month after booster vaccination with the Takeda vaccine was detected in 18 month old infants (Thomas, 1990).

Other Potential Vaccine Components: Several other potential protective antigenic components of *B. pertussis* have been shown to produce antibodies in recipients of whole-cell vaccines (Ashworth, 1983) but their importance in protection is uncertain and none as yet have been included in acellular vaccines. These include individual outer membrane proteins; heat labile toxin (HLT), tracheal cytotoxin (TCT) and adenylate cyclase toxin (ACT), and lipopolysaccharide endotoxin (LPS)..

ACT is known to inhibit both macrophage and lymphocyte metabolism (Confer & Eaton, 1982; Hewlett *et al.*, 1983b). There are no data on protective effects of anti-ACT antibody. Its role in the pathogenesis of pertussis and the generation of protective antibodies in animals or humans is largely unknown. ACT should be considered as a candidate for future evaluation when sufficient quantities of purified material are available for testing.

In the mouse intracerebral challenge test, purified HLT preparations had no protective activity (Nakase *et al.*, 1969). HLT has been considered not to be a protective antigen against whooping cough (Munoz, 1971; Munoz & Bergman, 1977; Pittman, 1979,1984) since vaccine preparations without HLT protect mice and children. Recent findings of the vasoconstrictive effects of the purified toxin on the peripheral blood supply to the respiratory tract have suggested that HLT may be involved in the initial stages of disease (Endoh *et al.*, 1986). To date TCT has not been vigorously pursued as a candidate antigen for an acellular vaccine. Endotoxin has antigenic properties but it does not appear to play a major role in protection.

Many components of *B. pertussis* are involved in the pathogenesis of disease but the role of the immune response to these components in prevention of colonization and disease in humans remains poorly defined (Cherry *et al.*, 1988; Mortimer, 1988; Oda *et al.*, 1985). While several components have been shown to protect mice against aerosol challenge, antibody studies in children to date have been unable to correlate the presence or titre of antibody against specific components with protection against the disease. Thus, the final selection of components for a vaccine that will afford protection against both infection and disease remains unresolved.

The exact quantity and proportions of each antigen to be incorporated into the vaccine will need to be studied in small immunogenicity and reactogenicity trials before a satisfactory formulation can be designed.

2.3.2 EXTRACTS OF *B. pertussis* CELLS AS VACCINES

Several groups have attempted to extract protective antigen, defined by activity in the ic mouse-protection test and several extract vaccines have been tested in field trials, none of which have come into general use. These vaccines are defined only on the basis of the same tests used to measure the potency and safety of whole-cell vaccines.

Pillemer *et al.* (1954) prepared a vaccine from sonicated *B. pertussis* cells which was used in

the MRC trials (1959) in the United Kingdom. Although the vaccine gave good protection, resulting in one of the lowest attack rates after exposure, it also gave more reactions than the whole-cell vaccine with which it was compared.

Pennell & Thiele (1951) made an ethanol treated sonicated extract vaccine which was used in a study by Felton & Verwey (1955). The vaccine produced reasonable levels of agglutinins. Another extract vaccine (Tri-Solgen, Eli Lilly) was tested for immunogenicity and reactogenicity by Weihl *et al.* (1963) and was extensively used during the period 1968-1974 (Robbins, 1984). More recently, a Behringwerke vaccine containing mainly OMPS of *B. pertussis* was tested in 3-4 month old infants but its reactogenicity was reported to be low (Schwick *et al.*, 1980).

2.3.3 DEFINED ACELLULAR VACCINES

Two types of defined acellular vaccine are currently available -

1. Preparations containing co-purified antigens in which the antigen content and ratios are fixed by the particular purification method used and cannot be independently varied.
2. Preparations containing individually extracted antigens in which the antigen content and ratios in the final blended product can be independently varied.

In addition to their antigen content, acellular vaccines also differ with respect to the toxoiding methods used and the nature of the toxoiding agent (formaldehyde, hydrogen peroxide, tetranitromethane, or glutaraldehyde). Both of these factors may affect immunogenicity and reactogenicity of the end product since residual toxicity may be present or reversion to toxicity may occur.

Several defined vaccines of varying composition are either in use or undergoing the various clinical trials which are prerequisites for new vaccines. These vaccines are summarised in Table

2.7 and discussed below.

The first generation of the new acellular vaccines was developed in Japan in the late 1970s and licensed for general use in that country in 1981. Because of demand for a new vaccine with minimum adverse reactions, Japanese acellular vaccine was quickly introduced into use, and the characterization of antigens was incomplete (Aoyama *et al.*, 1988).

JAPANESE VACCINES: The Japanese vaccines consist primarily of detoxified PT and FHA, either separately or in various proportions (but with some degree of contamination with other antigens) and are based on the experimental procedure described by Sato *et al.* (1984). Initially the ratio of FHA to PT in the Japanese vaccines was variable because the components were not separately purified and then blended together, but were co-purified. Two general types of acellular pertussis vaccines are produced in Japan; the B- and the T-type.

a. Takeda 'T'-type vaccine consists of FHA, PT, AGG-2 in the weight ratios of 90:10:1.

b. Biken 'B'-type vaccine which consists of equal proportions of FHA and PT (5 to 6 mg of protein nitrogen per litre), with no detectable amounts of AGGs or other substances.

Both types have been shown to produce fewer local and febrile reactions than whole-cell vaccines and therefore are considered safer (Aoyama *et al.*, 1986; Olin, 1986; Lewis *et al.*, 1985,1986). The B-type vaccine was chosen for routine use in Japan in 1981 following comparative hospital-based trials because T-type vaccines were found to be of varying purity. Furthermore, mean anti-PT and anti-FHA titres were greater after administration of B-type vaccine than after T-type or whole-cell vaccines (Noble *et al.*, 1987). More than 40 million doses have been administered to date (Kimura & Kuno-Sakai, 1990). The low toxicity of Japanese acellular vaccine in various laboratory tests was confirmed by a WHO collaborative study involving several laboratories (WHO, 1984).

Each lot of vaccine must meet the minimum requirements for potency and safety of the National Institute of Health, Japan. The vaccine is available from 6 different Japanese manufacturers, 5 of whom produced T-type vaccine (Takeda, Kita Sato, Chiba, Denka, Kaketsu). BIKEN (The Research Foundation for Microbial Diseases of Osaka University is the only producer of the B-type vaccine (Aoyama *et al.*, 1988). Variations in composition between the products of these manufacturers occur, however all contain at least 0.5 μg protein nitrogen of PT per ml and > 2.5 μg protein nitrogen of FHA per ml; and are considered to be equivalent by the Japanese government (Sato & Sato, 1988).

There are significant differences in the antibody response to the 2 types of acellular vaccine because of differences in content of pertussis antigens. The B-type vaccine produces significantly higher anti-PT responses than the T-type, while the B-type produces no agglutinin response (Aoyama *et al.*, 1986).

Studies on heat stable pertussis vaccine are now under way in Japan. This would prove useful in tropical and developing areas (Kimura & Kuno-Sakai, 1990).

JAPANESE NATIONAL INSTITUTE OF HEALTH PT MONOCOMPONENT VACCINE: Safety and immunogenicity trials were carried out in Sweden in adults and 5-11 month old infants (Hedenskog *et al.*, 1987). The vaccine was found to be highly immunogenic and associated with few mild adverse reactions.

LEDERLE/TAKEDA VACCINE (PT:FHA: 69kDa OMP 3:35:0.6): Studies in 4-6 year olds with this vaccine compared to whole-cell vaccine showed fewer local side effects within 72 hours in the former. Between 72 hours and 14 days post-vaccination, the overall rate of side effects was very low, and equal in both groups (Table 2.8). Anti-FHA levels measured by ELISA were greater in acellular vaccinees, however the levels of other antibodies were not significantly

different. A second study of the vaccines in 17-24 month old infants showed similar results as the 4-6 year old group except that the rate of fever within 24 hours of vaccination was greater in acellular vaccinees. A third series with vaccination at 2, 4 and 6 months and booster at 18 months showed fewer local and systemic reactions than in whole-cell vaccine recipients, greater response to FHA and equivalent response to 69 kDa OMP (Blumberg *et al.*, 1991).

CAMR VACCINE: This vaccine was developed in the United Kingdom and consists of equal amounts (10 μg/dose) of separately purified PT, FHA and AGG2,3 (in proportions of 3:2), each of which is formaldehyde treated, and then combined and adsorbed onto an aluminium adjuvant (Robinson *et al.*, 1986). The vaccine had low protective potency in mice against ic infection but offered protection against in infection with *B. pertussis*. In comparison with an adsorbed whole-cell vaccine, this vaccine produced in mice equivalent or reduced levels of antibody to FHA but much enhanced levels of antibody to PT and AGG2,3. In a phase I trial for reactogenicity and immunogenicity in adult volunteers the vaccine had low reactogenicity and induced high antibody responses to FHA, PT and AGG2,3. A Phase II trial of the vaccine in infancy showed fewer side effects and higher PT and AGG2,3 antibody titres than in whole-cell vaccinees (Miller *et al.*, 1991).

SMITH-KLINE BIOLOGICALS ACELLULAR PERTUSSIS VACCINE: This vaccine, containing PT and FHA, became available for clinical studies in 1988. The safety of a formulation containing 25 mcg of both PT and FHA was demonstrated in adult volunteers (Ruuskanen *et al.*, 1988) and 15 month old babies (Just *et al.*, 1987) who presented with a low rate of local and general side effects after receiving one dose.

A third vaccine component, 69 kDa outer membrane protein became available in 1990 and was administered in combination with PT, FHA, diphtheria and tetanus as a booster in adults and in 15 months old infants (Ruuskanen *et al.*, 1991). Primary vaccination studies with 3 consecutive doses in infants have been initiated.

MÉRIEUX (MONOCOMPONENT PT vs. PT:FHA): The vaccines produced by Mërieux (separately purified and glutaraldehyde-detoxified PT, alone or with equal amounts of FHA) produced significant local reactions but induced excellent antibody responses in adults. Studies of these vaccines in 3-6 month old infants resulted in a better serologic response in the vaccine containing both PT and FHA. Side effects to PT were not related to the number of injections. One year later higher antibody levels were recorded than in whole-cell vaccinees.

OTHER ACELLULAR PERTUSSIS VACCINES: A number of other acellular preparations have been produced by various manufacturers and are at different stages of development.

i. A vaccine developed by 'Connaught' was basically a Takeda-type vaccine, but detoxified by glutaraldehyde. It produced fewer local and systemic reactions than whole-cell vaccine but did not appear to be highly immunogenic (Table 2.9).

ii. 'Sclavo' were the first to derive a toxin with mutations in the S_1 subunit, thus producing a non-toxic toxin, hence no inactivation is required in production of the vaccine.

iii. A phase I trial of an 'Amvax' monocomponent vaccine consisting of PT detoxified with hydrogen peroxide was reported by Sekura *et al.*, 1988. Relatively high doses of the vaccine were of low reactogenicity in adult volunteers and induced a good antibody response to PT.

2.3.4 SUBUNIT OR SYNTHETIC PEPTIDE VACCINES

Recent advances in molecular biology have lead to a new approach to the production of vaccines. The development of safe and effective pertussis vaccine may be accelerated by the use of recombinant DNA technology. The complete nucleotide sequence of the PT gene has been determined and the amino acid sequence of all 5 subunits of the toxin have been deduced (Black & Falkow, 1986; Locht & Keith, 1986; Nicosia *et al.*, 1986), Rappuoli, 1986). The cloning and expression of the FHA gene has also been reported (Brown & Parker, 1986).

Diagrammatic representation of gene cloning is depicted in Figure 2.3. Manipulation of the PT genes by genetic engineering could result in the production of large amounts of defined specific antigen, free from extraneous material and thus should lead to effective, less reactive and more acceptable vaccines.

The ideal vaccine should contain a non-toxic and immunogenic PT molecule that does not require chemical treatment. Site-directed mutagenesis of the PT gene has resulted in a mutant inactive non-toxic PT fragment (Pizza *et al.*, 1989) without affecting immunogenicity and protectivity. This new molecule is biochemically and immunologically indistinguishable from wild-type PT but is at least 1 million times less toxic and may be the basis of a viable vaccine candidate in the future (Moxon & Rappuoli, 1990).

Genetic analysis of *B. pertussis* fimbriae is being undertaken with the aim of producing peptides which induce an immune response effective at blocking the adhesion of *B. pertussis* to mammalian cells (Robinson & Ashworth, 1988). The complete amino acid sequence of AGG-2 has been determined (Livey *et al.*, 1987).

The production of vaccines by genetic engineering has the advantage of yielding extremely pure vaccines, presumably at a lower cost and at a faster pace than is the case for those prepared by present techniques.

2.3.5 MANUFACTURE OF ACELLULAR PERTUSSIS VACCINES

Japanese acellular vaccine is prepared by the method of Sato *et al.* (1984) from a fraction of the culture supernatant of *B. pertussis* strain Tohama (serotype 1,2,4) phase I bacteria cultured in Cohen-Wheeler or Stainer-Scholte liquid medium at 35°C for 5 days. The fraction of culture filtrate obtained containing mainly PT and FHA (bacteria-free) is used as starting material for vaccine production.

The filtrate is concentrated and purified by salt (ammonium sulphate) fractionation and extracted with a high concentration of sodium chloride. Non-protective substances are partially removed by zonal ultracentrifugation or chromatography. The bulk material then undergoes superpurification, ie. endotoxin (pyrogen) is removed (Sato *et al.*, 1984) and the antigens are fractionated by sucrose density gradient ultracentrifugation. Electron microscopy, assays for endotoxin, and polyacrylamide gel electrophoresis (PAGE) are used to determine purity during the purification process. Fractions of the purified bulk material are then isolated, pooled and treated with formaldehyde to detoxify PT and FHA and combined with diphtheria and tetanus toxoids. Different manufacturers use glutaraldehyde, hydrogen peroxide or tetranitromethane to inactivate PT. Finally, aluminium hydroxide or aluminium phosphate adjuvant is added to produce the vaccines with final protein nitrogen content in the region of 15 μg/ml. Gelatin and glucose are added as stabilizers. Several *in vivo* and *in vitro* tests are then used to evaluate vaccine potency and toxicity. Compositional variations between products depend on modifications of media or culture conditions used by some manufacturers and variations in purification steps or conditions of formaldehyde inactivation. Figure 2.4 shows differences in preparation procedures of acellular vaccine from that of whole-cell vaccine.

2.3.6 LABORATORY EVALUATION OF ACELLULAR PERTUSSIS VACCINES

Several *in vivo* and *in vitro* systems have been developed for both potency (immunogenicity) and toxicity (safety) testing of candidate acellular pertussis vaccines and their component antigens but none to date have been endorsed by the WHO or by the Bureau of Biologics in the United States. Quality control is presently being made on the basis of as many as 11 different tests. The tests employed characterise acellular pertussis vaccines with respect to their component antigens, safety, potency and stability and where appropriate, comparison is made with highly purified preparations of pertussis antigens used in WHO collaborative studies.

Emphasis is given to estimating antigen content, purity, toxicity and immunogenicity. The tests

include limulus amoebocyte lysate assay (LAL) (for endotoxin), ELISA, analytical sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and electron microscopy. The same criteria for freedom from toxicity (histamine sensitization, Chinese Humster Ovary (CHO) cell, leucocytosis and ADP ribosylation) are applied to acellular and whole-cell vaccines although stricter requirements are applied to acellular vaccines (Kurokawa, 1984). Stability of the vaccine is tested by incubating at 37°C for prolonged periods and the measuring residual toxicity. The immunogenicity of toxoided antigens is tested by ELISA and CHO cell neutralisation assays (for PT). The value of the ic challenge mouse test (used to measure potency of whole-cell vaccines) in assessing acellular vaccines is in doubt.

Before any scheme for laboratory evaluation of the potency and safety of acellular pertussis vaccines is adopted as routine, correlation with performance in infants needs to be established.

2.3.7 VACCINE USAGE

Schedule and dosage: In Japan acellular vaccine is administered to children beginning at 2 years of age, with 3 doses of 0.5 ml given by deep subcutaneous injection at intervals of 3-8 weeks between doses. An additional single booster dose of 0.5 ml is administered between 12 and 18 months after the primary vaccination course.

Contraindications: The administration of this vaccine is contraindicated, (except when withholding it entails even greater risk) in the event of severe febrile illnesses or other active infection; illnesses of heart, kidney, liver; diabetes; malnutrition; leukaemia, lymphomas and other generalised malignancies; hypersensitivity and pregnancy.

2.3.8 ADVERSE REACTIONS

Local and systemic reactions: Local and systemic reactions to acellular vaccine have been studied in Japan, Sweden and the United States (*Ad hoc* group for the study of pertussis vaccines, 1988; Anderson *et al.*, 1987,1988; Aoyama *et al.*, 1986; Blennow *et al.*, 1988b,1989;

include limulus amoebocyte lysate assay (LAL) (for endotoxin), ELISA, analytical sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and electron microscopy. The same criteria for freedom from toxicity (histamine sensitization, Chinese Hamster Ovary (CHO) cell, leucocytosis and ADP ribosylation) are applied to acellular and whole-cell vaccines although stricter requirements are applied to acellular vaccines (Kurokawa, 1984). Stability of the vaccine is tested by incubating at 37°C for prolonged periods and then measuring residual toxicity. The immunogenicity of toxoided antigens is tested by ELISA and CHO cell neutralisation assays (for PT). The value of the ic challenge mouse test (used to measure potency of whole-cell vaccines) in assessing acellular vaccines is in doubt.

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(Edwards *et al.*, 1986,1989; Lewis *et al.*, 1985,1986). The occurrence of fever ≥38°C following Japanese acellular pertussis vaccination has ranged between 5-10% and from 30% to 85% following whole-cell vaccine. The reported frequency of fever ≥40.5°C (and hence vaccine-associated febrile convulsions) has ranged from 1% to 6.3% and appears to be less frequent in acellular vaccines.

Neurological reactions: It has been suggested but not proven that acellular vaccines are associated with fewer CNS reactions. Controlled data of the association of acellular vaccines and neurologic adverse events is not available. The Japanese compensation system for vaccine damage paid out claims for severe neurologic damage with sequelae at a rate of 1.39 per million doses when whole-cell vaccine was administered from 3 months of age. A reduction in rate was brought about by a change in the age of vaccination. Severe reactions and deaths per million doses occurred at approximately the same low rate (0.4 vs. 0.25/million doses) when either whole-cell or acellular vaccine were given at 2 years of age, suggesting a temporal rather than causal relationship between DTP administration and encephalopathy (Noble *et al.*, 1987) (Table 2.11). The risk of temporally related encephalopathy following acellular vaccine administration at 2, 4 and 6 months, the age of primary vaccination remains unresolved.

In 1985-1987 in a Swedish field trial of 4,000 infants aged between 5-11 months 4 deaths from invasive bacterial infections occurred in recipients of Japanese acellular vaccine during the first year of life and none in recipients of placebo were reported (*Ad hoc* Group for the Study of Pertussis Vaccines, 1988; Storsaeter *et al.*, 1988). Several investigations were carried out to examine the possibility that the deaths could be causally related to vaccination. There were no clinical or biological indications of increased susceptibility to invasive disease caused by encapsulated bacteria (Storsaeter *et al.*, 1988). Also, equal fatality and distribution over time in participating and non-participating countries suggest no causal relationship.

The expected rate of occurrence of hypotonic-hyporesponsive episodes (collapse, shock) in

infants could not be predicted from Japanese data because this illness occurs predominantly during the first year of life (Cody *et al.*, 1981). The use of acellular vaccine does not appear to eliminate the occurrence of these reactions (Blumberg *et al.*, 1991). Since available data do not support a causal relationship between onset of infantile epilepsy; sudden infant death syndrome, and whole-cell vaccination, a change to acellular vaccine cannot be expected to lower the rate of these reactions. Japanese researchers could find no evidence of increased age-specific mortality rates of meningitis or sepsis after the introduction of acellular vaccines (Kimura & Kuno-Sakai, 1988).

The assessment of the incidence of serious rare reactions following acellular pertussis vaccines requires further prospective studies and a prolonged surveillance programme.

2.3.9 PATHOGENESIS OF ADVERSE REACTIONS

Detoxified PT in acellular vaccines may in time revert to the active form since residual formalin used in the detoxification process is removed from the final vaccine product. Acellular vaccines may therefore be histamine-sensitizing and associated with CNS reactions and encephalopathies as a result of toxic PT (Kimura & Hikino, 1985).

2.3.10 CURRENT EXPLORATIONS OF EFFICACY

Acellular pertussis vaccines comprising PT and FHA has been routinely used in Japan since 1981. It has however proved difficult to draw firm conclusions about the protective efficacy of the vaccines as their introduction coincided with a change in the vaccination schedule. Efficacy in infancy has not been evaluated conclusively. Data are limited by the vaccines having been given mostly to 2 year old children; only recently have infants been vaccinated (Sato *et al.*, 1984; Noble *et al.*, 1987; Miller *et al.*, 1991; Blumberg *et al.*, 1991)). Furthermore, most of the data available are not specific for a particular vaccine product.

Acellular vaccines appear to have good efficacy in a number of epidemiological and clinical

studies in Japan (Kimura & Hikino (1985); Kimura & Kuno-Sakai, 1990; Isomura, 1988; Isomura *et al.*, 1985). A steady decline in disease and death due to pertussis has occurred in Japan since the vaccines were introduced in 1981. Epidemics have remained under control since the exclusive use of the vaccines.

In several retrospective Japanese household contact studies between 1978 and 1988, the efficacy of acellular vaccines in protecting children aged 1 year or older against clinical pertussis ranged consistently between 77-89% despite methodological differences (Sato *et al.*, 1984; Aoyama *et al.*, 1985; Isomura *et al.*, 1985; Kimura & Hikino, 1985) (Table 2.12). PT and FHA antibody levels were found to be equivalent to those in children recovering from pertussis (Sato *et al.*, 1984; Biritwum *et al.*, 1985; Isomura *et al.*, 1985; Sato & Sato, 1985).

In 1985, a group of United States scientists visited Japan to obtain information on the efficacy and safety of acellular pertussis vaccines (Noble *et al.*, 1987). The main conclusions were that the Japanese vaccines could be considered safe and effective in 2 year old children, but that additional data on safety and efficacy in infants was required.

Date obtained from trials of Japanese vaccine in the United States (Lewis *et al.*, 1985,1986; Edwards *et al.*, 1986) confirmed the immunogenicity and low reactogenicity in older children; and demonstrated comparable efficacy to conventional vaccines (Edwards *et al.*, 1986; Sato *et al.*, 1984; Aoyama *et al.*, 1988). More recently defined acellular vaccines prepared from separately purified and inactivated FHA and PT have shown good immunogenicity and low reactogenicity in a number of clinical trials.

These observations triggered the organisation of a series of carefully designed international collaborative efficacy trials in Sweden to determine the safety, efficacy and immunogenicity of Japanese acellular vaccines under conditions normally used for DTP vaccination in the Western world. Sweden was chosen because of the high attack rate of the disease, because whole-cell

DTP was not used routinely and because the clinical surveillance systems enabled 100% coverage. The Swedish study consisted of 3 phases: Phase I involved a small investigation in adults (Granström *et al.*, 1987); Phase II conducted in 1984-1985, studied the serological response and incidence of side effects after acellular vaccine in infants aged 6 months or older (Blennow *et al.*, 1988) and Phase III, a large safety and efficacy trial, was carried out in 1986-1987 in 5-11 month old infants (*Ad hoc* Group for the Study of Pertussis Vaccines, 1988). The infants received a placebo or 1 of 2 acellular pertussis vaccines containing PT only (JNIH-7) or in combination with the same amount of FHA (JNIH-6) (Olin, 1986).

Overall VE (based largely on clinical diagnosis of pertussis) was found to be 69% in JNIH-6 recipients and 59% in recipients of JNIH-7; lower than that of many currently used whole-cell vaccines. Both vaccines induced good antibody responses to the vaccine antigens, however post-vaccination serum PT and FHA antibody levels did not correlate with clinical protection, although both vaccines gave protection against severe disease (*Ad hoc* Group for the Study of Pertussis Vaccines, 1988; Storsaeter *et al.*, 1988). Recently post-trial surveillance data were used to recalculate efficacy data (Olin *et al.*, 1989). $\widehat{VE}$ against culture-proven pertussis disease was 83% with PT-FHA and 72% with PT alone.

The results suggest that the less specific the definition of whooping cough, the lower the $\widehat{VE}$. The efficacy of both vaccines tested in the Swedish trial appeared to be lower than expected. Despite the demonstration of some protection, especially against severe clinical manifestations, the data are not generally accepted and did not lead to acceptance or licensing of the vaccines for routine vaccination (Olin *et al.*, 1989).

The reduced clinical efficacy and lack of correlation between antibody levels and protection suggest that additional components of *B. pertussis*, present in the conventional vaccine and absent in acellular vaccines may play a role in disease protection.

Several other investigations of acellular preparations are currently being undertaken. A further United States National Institute of Health Phase III protection trial will probably take place in Sweden in 1992. The efficacy of 2 acellular vaccines; 1 containing only PT and a second complex vaccine containing 2 or more antigens will be compared with a whole-cell vaccine preparation and a placebo.

Further clinical trials on the efficacy and safety of acellular pertussis vaccines are needed in other countries to support results of the Swedish study before the vaccines can be integrated into existing schedules. The ultimate goal is a vaccine of efficacy equal to or exceeding levels of current whole-cell vaccines, with a high level of safety in infants and one that will permit safe vaccination of older individuals including pregnant women.

TABLE 2.1 Adverse events occurring within 48 hours after administration of whole-cell pertussis vaccine (summary of the data produced in several studies)**.

EVENT	FREQUENCY*
Local	
Redness	1/3
Swelling	2/5
Pain	1/2
Mild/moderate systemic	
Fever > 38°C	1/2
Drowsiness	1/3
Fretfulness	1/2
Vomiting	1/15
Anorexia	1/5
More serious systemic	
Persistent (> 3 hours) unconsolable crying	1/100
High pitched unusual screaming	1/900
Fever > 40.5°C	1/330
Hypotonic-hyporesponsive episode	1/1750
Convulsions (with or without fever)	1/1750
Acute encephalopathy +	1/110,000
Permanent neurologic deficit +	1/310,000

* Number of adverse events per total number of doses regardless of dose number in DTP series.

** Recommendations of the Immunization Practices Advisory Committee, 1985; Manclark *et al.*, 1984; Miller *et al.*, 981; Pallock *et al.*, 1984; Cody *et al.*, 1981; Barkin & Pichichero, 1979).

\+ Occurring within 7 days of DTP vaccination.

TABLE 2.2 Estimated neurological reaction rates following whole-cell DTP vaccination as compared to complications of natural whooping cough (after Galazka *et al.*, 1984).

ADVERSE REACTION	RATE OF COMPLICATIONS COMPLICATIONS PER 100,000 CASES OF PERTUSSIS DISEASE	ADVERSE REACTION PER 100,000 DOSES OF OF WHOLE-CELL PERTUSSIS VACCINATION
Permanent brain damage	600-2000 (0.6-2.0%)	0.2-0.6
Encephalopathies/ encephalitis*	90-4000 (0.09-4.0%)	0.1-3.0
Convulsions	600-8000 (0.6-8.0%)	0.3-90
Shock	-	0.5-30
Death	100-4000 (0.1-4.0%)	0.2

* Including seizures, focal neurological signs, coma, Reye's Syndrome.

TABLE 2.3 Frequency of convulsions following whole-cell pertussis vaccination.

AUTHORS	YEAR	INCIDENCE OF CONVULSIONS
MRC	1956	1/11,000
Strom	1967	1/6,500
Griffith	1978	1-3/100,000
Hannick & Cohen	1979	1/2,750
Koplan	1979	1/1,250
Cody *et al.*	1981	1/1,750
Miller	1983	1/3,500
WHO	1984	0.6-8/100

TABLE 2.4 Frequency of states of shock following whole-cell pertussis vaccination.

AUTHORS	YEAR	INCIDENCE OF SHOCK/COLLAPSE
Hopper	1961	4/5,100
Strom	1967	1/6,500
Haire	1967	5/969
Hannick & Cohen	1979	4/11,000
Cody *et al.*	1981	9/15,752 (1/1,750)
WHO	1984	0.5-30/100,000

TABLE 2.5 Summary of estimates of the efficacy of conventional pertussis vaccines as reported in the literature. (Adapted from Fine & Clarkson, 1987).

TYPE OF STUDY (Author)	PLACE	YEAR(s)	VACCINE EFFICACY (%)
Controlled trial			
MRC	UK	1946-1950	61; 69; 72; 86; 91
Bell	USA	1938-1941	70
Cohort			
Madsen	Denmark	1923	≈0
		1929	24
		1919	94
Ditchburn	UK	1977-1978	4
Wilson *et al.*	UK	1964	10; 68; 13; 2
McFarlan *et al.*	UK	1942-1944	11
Noah	UK	1972-1974	60
Bousfield & Holt	UK	1953-1954	66; 87
MacGregor	UK	1977-1978	68
Kendrick & Eldering	USA	1934-1937	80
PHLS	UK	1978-1980	83; 93
Laurell *et al.*	Sweden	1956	88; 96; 100
Basilli & Stewart	UK	1974	0.72
Kendrick & Eldering	USA	1932-1936	91
Church	UK	1978	89
Cziser	Hungary	1970-1973	>97
Jenkinson	UK	1977-1978	88
Secondary attack rate			
PHLS	UK	1966-1968	53
	UK	1978-1980	51; 81
Royal College of General Practitioners	UK	1977-1979	41
Lambert	USA	1962	54
Brink *et al.*	USA	1979-1981	64
Grob *et al.*	UK	1978	58; 73
WHO/EPI	New Zealand	1982	59
Broome *et al.*	USA	1977	54
MRC	UK	1946-1950	66; 67; 72; 90; 91
TANAKA	Japan	1975;1977	69
CDC	US	1979-1981	82
	US	1982-1983	91
Broome *et al.*	US	1979	93
Stewart	UK	1978	45

TABLE 2.5 (Continued)

TYPE OF STUDY (Author)	PLACE	YEAR(s)	VACCINE EFFICACY (%)
Case control			
Trollfors & Rabo	Sweden	1978	0
WHO/EPI	New Zealand	1982	0
Stewart	UK	1978	61
Public Health Lab	UK	1963-1964	64; 80
Malleson & Bennet	UK	1964-1974	79
Johnston *et al.*	UK	1970-1979	84
Preston & Stanbridge	UK	1969-1971	90
Weiss & Kendrick	USA	1936-1941	84
British Society for Study of Infection	UK	1965-1974	94
Preston	UK	1974-1975	95
Walker *et al.*	UK	1969-1980	95
	USA	1979-1981	96
Smith *et al.*	UK	1978-1979	97

TABLE 2.6 Candidate molecules for acellular pertussis vaccines. (After Moxon & Rappuoli, 1990).

VIRULENCE FACTOR	ROLE IN PATHOGENESIS	MOUSE PROTECTION IC*	AEROSOL
Filamentous haemagglutinin	Bacterial adhesion	-	+ +
Agglutinogens 2 and 3	Bacterial adhesion	-	+ +
69 outermembrane protein	Bacterial adhesion	+/-	+ +
Pertussis toxin	Systemic toxicity; bacterial adhesion	+ +	+ +
Adenylate cyclase	Evation of host defences; virulence	ND	ND
Tracheal cytotoxin	Damage to ciliated cell	ND	ND
Dermonecrotic toxin	Local damage	ND	ND
Haemolysin	Local damage	ND	ND

ND not done
* intracerebral

TABLE 2.7 STATUS OF DEFINED ACELLULAR VACCINES.

CANDIDATE VACCINE	ANTIGENIC COMPOSITION (ratio)	TOTAL PERTUSSIS PROTEIN/DOSE	TOXOIDING AGENT	ANTIGEN PREPARATION	STAGE OF DEVELOPMENT
PHLS/CAMR (T-type)	PT/FHA/AGG2+S (1:1:1)	30 ug	Formaldehyde	Individual purification	Clinical studies in adults
TAKEDA/ CYANAMID	PT/FHA/AGG2 (90:10:1)	47.5 ug	Formaldehyde	Co-purified	4-6 yr (used to immunise children >2 yr in Japan since 1981)
CONNAUGHT	1. PT/FHA (T-type) (6:1) 2. PT/FHA/AGG/69KOMP	25 ug	Glutaraldehyde	Co-purified	Clinical studies: 17-19 mth old infants,
BIKEN National Institute of Health, Japan	1. PT (JNIH7) 2. PT:FHA (1:1) (JNIH6)	75 ug	Formaldehyde Formaldehyde	Individual purification	1. Adult: Clinical studies: 18 mth infants in Sweden 2. Used to immunise children >2 yr in Japan since 1981
Merieux	1. PT 2. FHA:PT (1:1)	25,50 or 12.5 ug 50 ug	Glutaraldehyde Glutaraldehyde	Individual purification	Clinical studies: adults & infants 18-24 mths-4-6 yrs, 3-6 mths, 18 mths
Lederle/ Takeda	1. PT/FHA/AGG/69KOMP (3:35:0-6) 2. PT-FHA-AGG (new DPT)			Co-purified Co-purified	Phase II 4-6 yr, 17-24 mth (problems with active toxin)
AMVAX	PT(H2O2)		Hydrogen peroxide (H2O2)	Individual purification	Sweden, clinical studies: adults
Massachusetts	PT TNM)		Tetronitromethane (TNM)	Individual purification	
Sclavo	1. PT (new). 2. PT (new)-FHA-69KOMP				
Smith Kline Biologicals	1. PT-FHA 2. PT-FHA-69KOMP	25 ug			Clinical studies: adults, 15 mth old babies

TABLE 2.8 Results of double-blind reactogenicity study using Takeda acellular pertussis vaccine in 18-24 month old children.

SYMPTOMS	ACELLULAR DTP (n=120)	WHOLE-CELL DTP (n=60)	P-VALUE FROM FISHERS' EXACT
Any adverse effect	55(46%)	52(87%)	<0.001**
Fever	6(05%)	12(20%)	0.003*
Appetite disorder	5(04%)	11(18%)	0.004*
Vomiting	1(01%)	3(05%)	0.109
Sedation	6(05%)	8(13%)	0.074
Crying/fretfulness/fussiness	5(04%)	19(32%)	<0.001**
Gait disorder	0	6(10%)	0.0012*
Injection site reaction	50(42%)	49(82%)	<0.001**

* and ** denote statistically significant differences between groups at the 0.01 and 0.001 levels respectively.

TABLE 2.9 Results of reactogenicity study with Connaught acellular pertussis vaccine in 18 month old children.

SYMPTOMS*	ACELLULAR (n=25)	WHOLE-CELL (n=27)
Fever ≥39°C	O (-)	1 (4%)
Persistent crying	1 (4%)	1 (4%)
Irritability	2 (8%)	10 (37%)
Drowsiness	0 (-)	4 (15%)
Eating less	0 (-)	7 (26%)
Local redness	3 (11%)	20 *74%)
Local swelling	1 (4%)	19 (70%)

* Evaluated at 24 hours.

TABLE 2.10 Results of Swedish double-blind reactogenicity study in six month old infants (Blennow *et al.*, 1985).

SYMPTOMS	PLACEBO* (n = 115)	ACELLULAR** (n = 115)	WHOLE-CELL*** (n = 73)
Fever > 37.5°C	40	52	75
Local reaction	16	19	74
Crying	3	8	34
Anorexia	3	3	9
Excess sleeping	15	13	29

* Carrier solution for acellular vaccine only
** PT/FHA vaccine
*** Wellcome plain pertussis vaccine

TABLE 2.11 Adverse events within 7 days of DTP immunisation in 2 year old children (based on claims paid by vaccine compensation system, Japan).

PER 10^6 VACCINE DOSES	WHOLE-CELL VACCINE 1975-1981	ACELLULAR VACCINE 1981-1984
Number of vaccine doses ($x10^6$)	19.8	20.4
Number of severe reactions with sequelae (number of deaths)	8	5
Incidence of severe reactions per million doses	0.40	0.25
Number of mild reactions without sequelae	34	14
Incidence of mild reactions per million doses	1.72	0.69 (till 1984)

NOTE: Modified from Noble *et al.*, (1987), Kimura & Kuno-Sakai, (1990).

TABLE 2.12 Efficacy of Japanese acellular pertussis vaccines in home-contact studies (source Noble *et al.*, 1987).

PERTUSSIS ATTACK RATE	STUDY A	B	C	D	E	TOTAL
Unvaccinated	59/72	17/20	83/121	28/34	14/18	201/265
Vaccinated	3/42	1/12	4/26	2/30	1/10	11/120
Vaccine efficacy	91%	90%	78%	92%	87%	88%
95% confidence intervals	(74-97)	(35-99)	(44-91)	(69-98)	(16-98)	(79-93)

A Kimura & Hikino (1985)
B Isomura *et al.* (1985) (Takeda vaccine)
C Aoyama *et al.* (1985)
D Isomura *et al.* (1985)
E Aoyama *et al.* (1985)
F Aoyama *et al.* (1988) (T-type) 88% (68-95); (B-type) 77% (0-96).

AGE (yrs)	AGE-SPECIFIC EFFICACY
1-2	83 (57-93)*
3-5	93 (80-98)
≥6	54 (0-83)

* 95% confidence intervals.

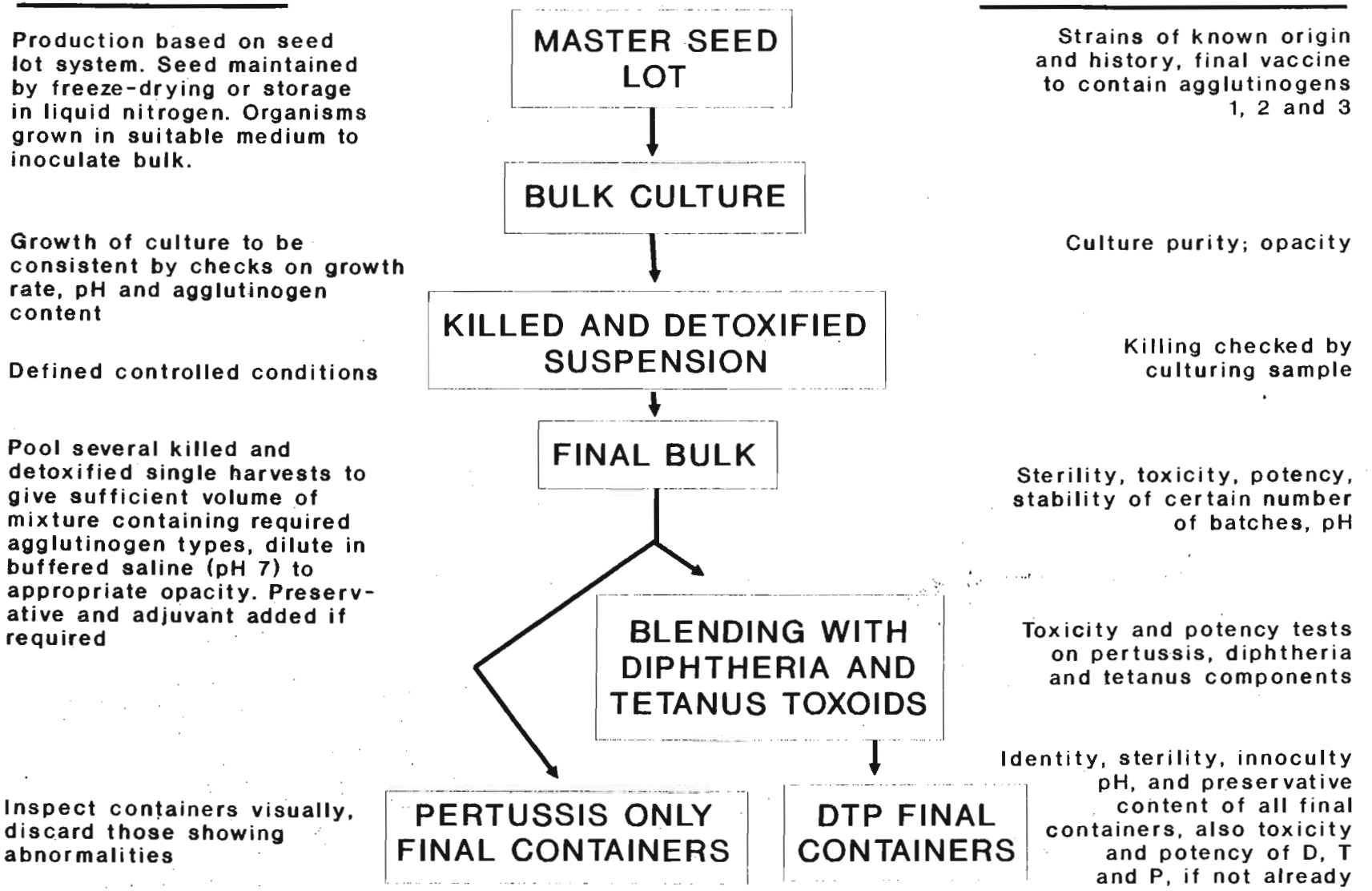


FIGURE 2.1 Production of whole-cell pertussis vaccine according to WHO (1979) specifications.

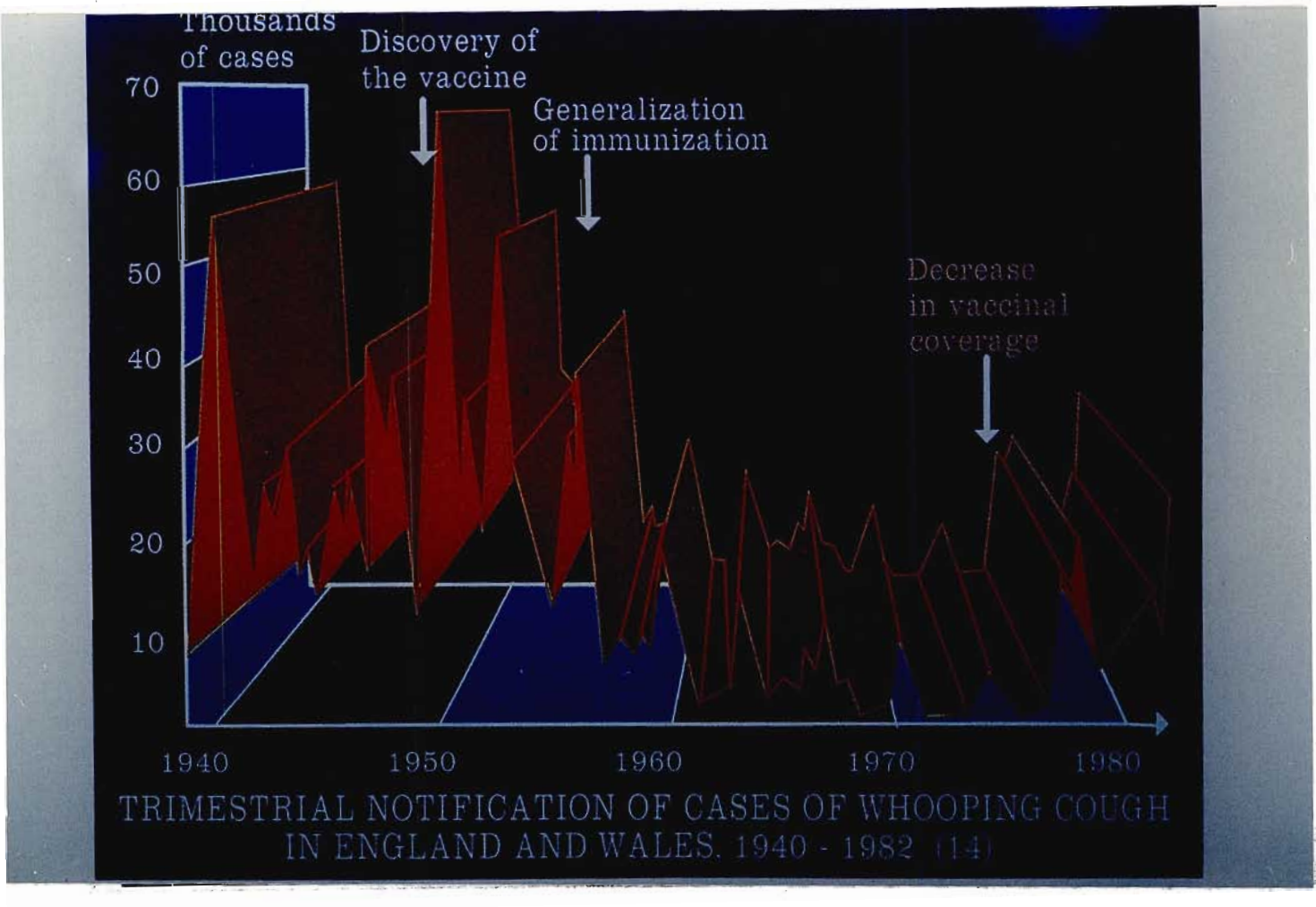


FIGURE 2.2 Effect of the implementation and large-scale application of a vaccination programme in England and Wales, 1940-1982.

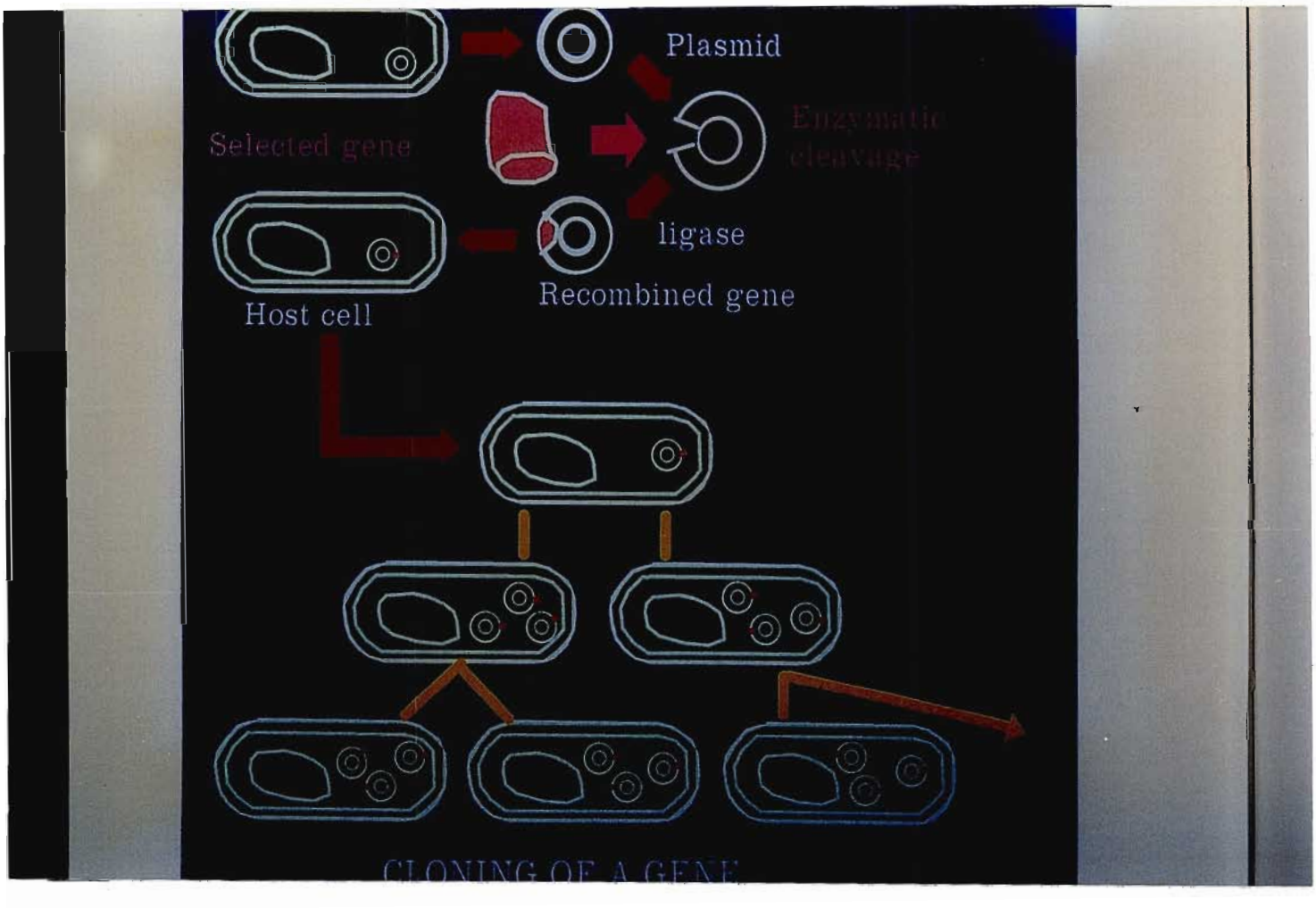


FIGURE 2.3 Diagrammatic representation of gene cloning.

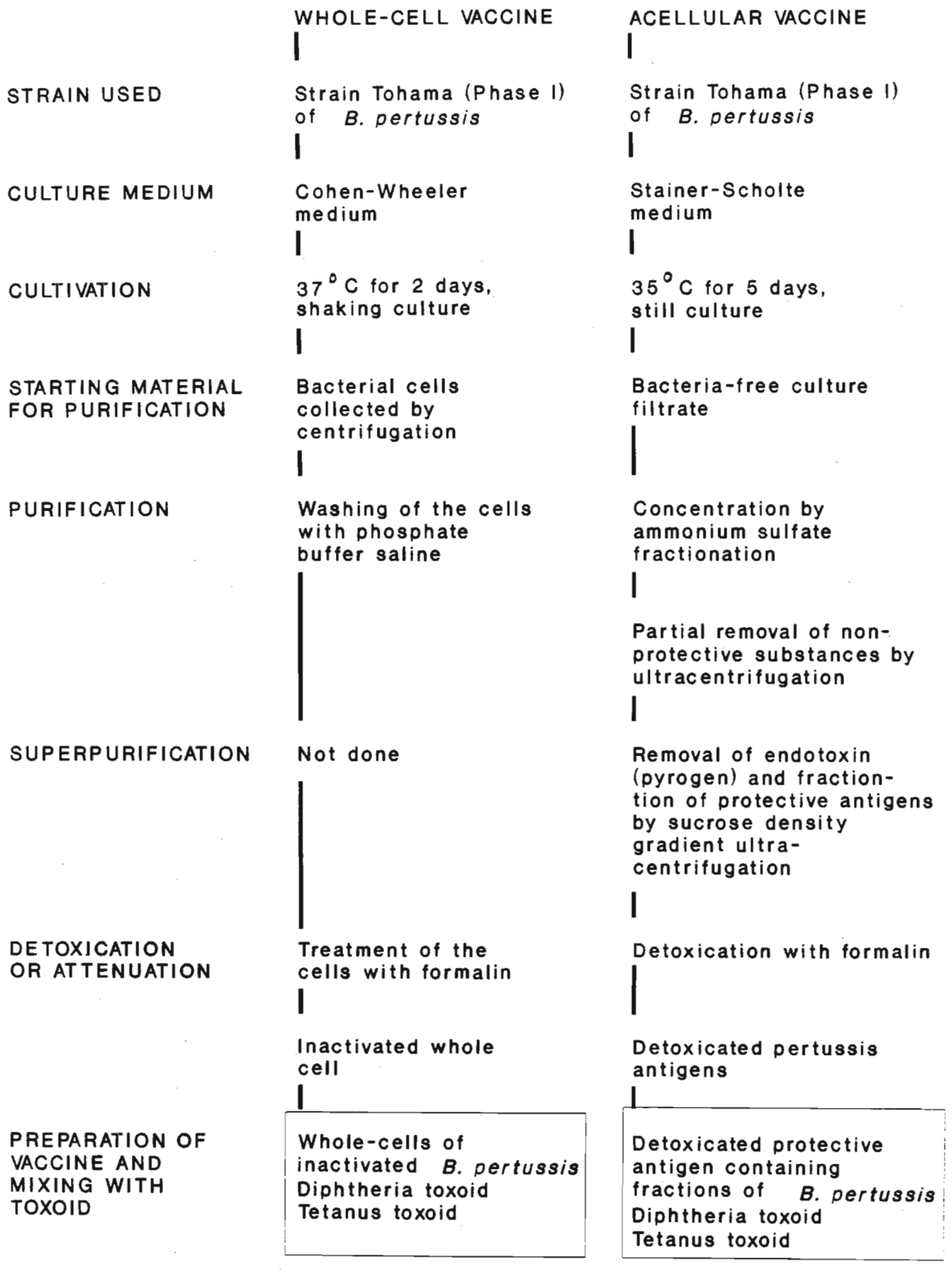


FIGURE 2.4 Differences in preparation procedures of acellular vaccine from that of whole-cell vaccine.

CHAPTER 3

SEROLOGIC RESPONSES TO *Bordetella pertussis*

3.1 INTRODUCTION

This chapter presents current knowledge of the role and measurement of serological and secretory antibodies in pertussis immunity and diagnosis. Both infection and vaccination provoke antibody responses against a variety of *B. pertussis* antigens. The role of these antibodies is not well understood. Antibody levels are generally higher and longer-lasting after infection than after vaccination (Linnemann, 1979).

IgG antibodies to specific *B. pertussis* antigens are produced as a response both to vaccination and to infection. In humans, secretory IgA anti-pertussis antibodies and serum IgA and IgM antibodies are produced as a response to natural infection but not to vaccination (Ruuskanen *et al.*, 1969; Nagel & Poot-Scholtens, 1983; Nagel *et al.*, 1985; Macauley, 1981), although the expected rise after infection may be muted in those previously vaccinated (Nagel *et al.*, 1985).

The differences in the assortment and levels of antibodies induced after vaccination and disease may explain the less durable protection obtained by vaccination. In both cases protection against symptomatic disease is greater than immunity to infection (Long, 1990b; Fine & Clarkson, 1987; Thomas, 1989; Mortimer, 1990). The study of possible qualitative and quantitative differences between the antibody spectrum in convalescents and vaccinated individuals may help to discern between essential immunogens and unimportant antigens.

The different antigens of *B. pertussis* to which serologic and other responses are measured include: PT, FHA, AGG2,3, lipopolysaccharide endotoxin, outer membrane proteins, heat labile protein, and adenylate cyclase toxin. Experimental evidence suggests that PT and FHA are the major antigens in the production of protective immunity to *B. pertussis*, however the acquisition of IgG antibodies to these antigens has not been shown to correlate to clinical immunity or vaccine efficacy. Agglutinating antibodies may play a supportive role. The significance of antibodies to pertussis specific ACT in protection has not been elucidated. The

role of cell-mediated immunity in pertussis has not been adequately evaluated.

To date no specific animal models or serologic procedure is available that has proved to be a direct measure of the hosts' immunity to infection or disease (Manclark & Cowell, 1984). Serologic responses following both pertussis infection and vaccination have been measured *in vitro* by bacterial agglutination assays (Kendrick *et al.*, 1969; Kendrick, 1933; Abbot *et al.*, 1971), precipitins (Aftandelians & Conner, 1973), bactericidal assays (Ackers & Dolby, 1972; Dolby & Stephens, 1973), counter immunoelectrophoresis (CIE) (Boreland & Gillespie, 1984), complement fixation test (British Medical Journal, 1970; Bradstreet *et al.*, 1972) and ELISA (Baraff *et al.*, 1983; Viljanen *et al.*, 1982; Rutter *et al.*, 1988) with varied but generally unsatisfactory results. IgG responses seem to be directed to a wide molecular-weight region of *B. pertussis* polypeptides. IgM seems to be selectively specific only to PT, IgA reactivity is mainly directed to FHA (Partanen *et al.*, 1986).

Respiratory tract infection of mice with *B. pertussis*, especially aerosol inhalation of the bacteria by suckling mice, is a useful model for studies on the pathophysiology and immunity of human pertussis.

3.2 ANIMAL MODELS FOR THE STUDY OF IMMUNE RESPONSES TO *B. pertussis*

The lack of an animal species in which *B. pertussis* induces the same disease as in man has resulted in incomplete knowledge of the mechanisms of pathogenesis and immunity to pertussis (Pittman, 1979; Sato *et al.*, 1980; Ashworth *et al.*, 1982).

Several animal models (mice, rabbits, rats, monkeys) and various routes have been described for experimental pertussis infection. Among them are the intraperitoneal route (Cohen & Scandron, 1943), the intratracheal route (Bradford, 1938; TePunga & Preston, 1958(, the

aerosol or intranasal (in) route (Burnet & Timmins, 1937; Standfast, 1958(, and the intracerebral (ic) route (Standfast, 1958; Kendrick *et al.*, 1947). The animal is vaccinated with the experimental antigen preparation and then challenged with virulent *B. pertussis* and tested for survival. Two most commonly used tests for assaying the potency of pertussis vaccines to date are in the intracerebral (ic) mouse protection test (ICMPT) and the intranasal (in) mouse protection test (INMPT).

The INMPT model was first described by Burnet & Timmins (1937). Aerosol or in inhalation of *B. pertussis* by mice results in respiratory tract infections which resemble pertussis in humans in several ways, viz. infection route, localisation and adherence of bacteria, age-dependency on severity of the disease, duration of clinical symptoms and acquired immunity to reinfection (Sato *et al.*, 1980; Pittman, 1979). This model is however not ideal since mice are killed by *B. pertussis* of AGG type 1 which is avirulent in man (Carter & Preston, 1981). Furthermore, no paroxysmal cough occurs in aerosol infected mice, nor is there bacterial transmission from mouse to mouse. The INMPT has been valuable in evaluating the protective activity of PT, FHA and AGG-2 which were not effective in the ICMPT However, there are no data on the correlation between vaccine potency and efficacy in infants in this model and it is unlikely that the INMPT will be introduced for the assay of any candidate antigens for acellular vaccines (Sato *et al.*, 1979, 1980; Pittman *et al.*, 1980).

The ICMPT has until recently been the only accepted procedure for evaluating antigens and antibodies for protective activity. The potency of whole-cell pertussis vaccines and of a crude acellular preparation (Pillemer vaccine) correlated well with protection in children in this test (Medical Research Council, 1959). The ic route of infection is however unnatural; furthermore, fresh disease isolates of *B. pertussis* are not virulent when injected by this route (Andersen, 1953; Standfast, 1958; Preston & Evans, 1963). In the ic-challenged mouse, the bacteria are found on the ciliated epithelium of the ependymal cells in the brain (Hopewell *et al.*, 1972),

whereas in man *B. pertussis* multiplies in association with the ciliated respiratory tract epithelium. The ICMPT does not take into account the serotype specificity of either the vaccine or challenge strains (Preston & TePunga, 1959; Preston, 1963; Preston & Evans, 1963; Preston & Garrity, 1967). Acellular pertussis vaccines appear to be inactive or only partially active in the ICMPT. As judged by this test, neither FHA nor antibody to FHA was protective (Oda *et al*, 1984; Sato & Sato, 1984). PT was found to be less potent than whole-cell vaccine, and only when one or more antigens were added did the potency of acellular vaccines approach those of whole cell vaccines (Sato *et al*., 1974; Munoz *et al*., 1981a, Novotny *et al*., 1985). Alternative methods of estimating vaccine potency are therefore indicated.

3.3 ANTI-ADHERENCE ANTIBODIES

Pertussis infection in man is initiated by specific adhesions of virulent phase I *B. pertussis* surface antigens to ciliary surfaces of respiratory epithelial cells (Matsuyama, 1977; Tuomanen & Hendly, 1983). Adhesion hence appears to be a critical determinant of host susceptibility to the disease. Consequently, circulating and locally produced anti-adherence antibodies to *B. pertussis* surface antigens may play an important role in inhibiting the adherence of *B. pertussis* to ciliated cells, and therefore in immunity to pertussis.

Serum IgG anti-adherence antibodies were demonstrated to be present in higher titres after infection than after vaccination (Tuomanen *et al*., 1984). IgG anti-adherence antibodies were also detected in cord sera and in sera from adults who had had pertussis in childhood. Both IgG and IgA anti-adherence antibodies have been identified in sera after natural infection whereas only IgG antibody was present in infants receiving pertussis vaccine. Immunisation may fail to elicit IgA antiadherence activity (Tuomanen *et al*., 1984). IgG with anti-adherence activity was found in convalescent sera and in adult sera with high anti-adherence activity, in sera of vaccinees, but not in cord sera, nor in sera from adults with low levels of adherence-

inhibiting activity. IgM with anti-adherence activity has not been detected.

3.4 ANTIBODIES TO INDIVIDUAL *B. pertussis* COMPONENTS

3.4.1 PERTUSSIS TOXIN

There is nearly universal agreement that PT is the essential protective antigen for immunising man against pertussis (Pittman, 1984), and that long-term immunity following clinical infection correlates with the level of antibodies to PT (Granström *et al.*, 1987).

Antibody responses to PT may be found in different classes of immunoglobulin in different individuals. IgG and IgM responses to PT are generally higher after infection than after vaccination. IgG-PT titres were observed to be significantly higher after disease than after both whole-cell vaccine (Winsnes *et al.*, 1985; Granström, 1985, Granström *et al.*, 1985), and Japanese acellular pertussis vaccine (Sato & Sato, 1985). Serum IgA-PT are produced after infection but not after vaccination (Burstyn *et al.*, 1983; Winsnes *et al.*, 1985).

The percentage of IgG and IgA-PT responders correlates with age, both after vaccination and after infection (Nagel *et al.*, 1985; Winsnes *et al.*, 1985). The PT seroconversion rate in older vaccinees was reported to be about twice that attained in infants 2-3 months of age. No age-relation was detected in the IgM-PT antibody responses. In a recent longitudinal study of naturally infected and exposed Swedish children, Zackrisson *et al.* (1990) suggest that antibodies to PT alone may be protective.

3.4.2 FILAMENTOUS HAEMAGGLUTININ

Serum IgG, IgM and IgA antibodies to FHA are significantly higher after infection than after vaccination and therefore are thought to play a role in short-term immunity by inhibiting bacterial adherence (Winsnes *et al.*, 1985; Granström *et al.*, 1982). They are not essential for

recovery and long-term immunity against pertussis as indicated by the fact that FHA antibodies are not uniformly detected in convalescents from the disease (Nagel *et al.*, 1985; Winsnes *et al.*, 1985); as well as by the rapid disappearance of anti-FHA antibodies in culture-verified cases (Granström, 1982). In follow-up studies in Sweden, 43% of infants with antibodies to FHA alone contracted the disease (Zackrisson *et al.*, 1990).

The percentage of pertussis patients responding with IgM, IgG and IgA anti-FHA antibodies has been reported to increase with age (Granström *et al.*, 1982; Winsnes *et al.*, 1985). No correlation was found between anti-FHA levels and duration of pertussis symptoms (Viljanen *et al.*, 1985). Higher serum IgG, IgM and IgA to FHA have been reported after infection than after whole-cell pertussis vaccination (Winsnes *et al.*, 1985). IgG-FHA antibody titres following acellular pertussis vaccine were reported to be higher than those obtained after infection (Sato & Sato, 1985). The attack rate after household exposure was 13% in this study, indicating the minor importance of IgG-FHA for protection against disease.

3.4.3 AGGLUTINOGENS (AGGs)

Three AGGs, 1, 2 and 3, are considered to be important in the epidemiology of pertussis (Olson, 1975). AGG-1 is species specific, while AGG2 and AGG3 are type-specific (Preston, 1966).

The protective role of antibodies to AGG2,3 remains unclear and has been reviewed by Wardlaw & Parton (1983). Administration of whole-cell pertussis vaccine or recovery from pertussis infection (which is known to confer prolonged immunity in most cases) may not always result in the production of agglutinating antibodies and immunity may exist in the absence of demonstrable agglutinins.

The relationship between immunity and agglutinating antibody titres is unclear although high titres ($\geq$ 1:320) have been correlated with protection from disease (Kendrick *et al.*, 1933; Miller

et al., 1943; Sako *et al.*, 1947; MRC 1956; Oda *et al.*, 1983; Miller, 1986). Conversely it has been reported that protection may occur in infants with low agglutinin responses (Butler *et al.*, 1962; Miller *et al.*, 1943; Sako, 1947). However, these studies used a variety of agglutination methods and cannot be related quantitatively.

Agglutinin responses to disease were lower and more irregular than responses to whole-cell pertussis vaccination (Miller & Silverberg, 1939; Winter, 1983). Transmission of agglutinins through human colostrum (Oda *et al.*, 1983) suggests a role in protection of newborns in the first months of life.

The production or presence of agglutinating antibodies in whole-cell vaccinees is indirect evidence of vaccine potency and has been shown to be related to the protective power of the vaccine (Evans & Perkins, 1953).

3.4.4 LIPOPOLYSACCHARIDE (LPS) ENDOTOXIN

LPS of *B. pertussis* is located in the cell envelope and is released as membrane blebs into the surrounding medium (Parker *et al.*, 1985).

B. pertussis LPS antibodies are responsible in part for nonspecific protection against viral and bacterial infections but are not thought to play a crucial role in immunity against pertussis in man (Pittman 1984; Robinson *et al.*, 1985).

Several authors have described the occurrence of anti-LPS antibodies after both pertussis infection and vaccination (Winsnes *et al.*, 1985). Markedly higher IgA and IgM-LPS antibodies than IgA and IgM PT and FHA antibodies were demonstrated in sera from infected children (Nagel *et al.*, 1985). IgG-LPS titres were found to be higher than IgG-FHA but lower than IgG-PT. Winsnes *et al.* (1985) found no pronounced difference in the IgA response to LPS in

vaccinated and infected patients.

3.4.6 OUTER MEMBRANE PROTEINS (OMPs)

OMPs are located on the surface of *B. pertussis* (Olson, 1975) and on membrane-bound extracellular organelles (Parker *et al.*, 1985). The protective activity of OMPs in the ICMPT (Robinson & Hawkins, 1983) and in the INMPT (Robinson *et al.*, 1985) was found to be dependent on the presence of low levels of active PT; possibly as a result of the pharmacological effect of PT in increasing the permeability of the blood-brain barrier. PT may also mediate adjuvant activity, stimulating the antibody response to the OMPs.

3.4.7 HEAT-LABILE TOXIN (HLT)

As reviewed by Munoz & Bergman (1977), HLT is not thought to be involved in immunity to pertussis since vaccine preparations without HLT protect mice and children and purified HLT preparations have no protective activity. Furthermore, anti-HLT titres in convalescent sera are very low, if present at all (Evans & Maitland, 1939).

3.4.8 ADENYLATE CYCLASE (AC)

Weiss *et al.* (1984) showed *B. pertussis* AC to be a virulence factor for in infection in suckling mice, and purified AC from the culture supernate of *B. pertussis* was found to be highly protective in mice given an ic challenge (Novotny *et al.*, 1985). The role of antibodies to *B. pertussis* AC in human immunity however remains to be determined.

3.5 HUMAN PERTUSSIS IMMUNOGLOBULIN

There continues to be controversy about the possible benefits of anti-pertussis serum or pertussis immunoglobulin when given either after exposure or after the start of the coughing (paroxysmal) stage. As reviewed by Johnsson & Lundström (1950) pertussis immunoglobulin

may give some protection if administered to infants before or shortly after exposure. No clinical improvement can be expected after the PT has bound to its receptor, ie. in the paroxysmal stage of the disease.

Imaizumi *et al.* (1985) reported the beneficial effect of both human normal immunoglobulin and rabbit anti-PT immunoglobulin preparations in mice challenged with *B. pertussis* by the aerosol route. Protective activity was correlated with the anti-PT titre. Pertussis immunoglobulin is however not recommended at present for post-exposure immunoprophylaxis.

3.6 ANTIBODIES IN SECRETIONS

In the newborn secretions contain traces of maternal IgG, but no IgA or IgM. IgG diffuses passively through epithelial cells or leaks through breaks in the mucosa into nasal secretions. IgG predominates throughout the first year of life, unless the infant experiences a respiratory infection (Cohen *et al.*, 1970).

Secretory IgA antibodies appear to play a major role in recovery from pertussis. They are detected in humans only after infection. The susceptibility of newborn infants to pertussis may be connected with a lack of IgA antibody however the importance of this antibody in long-term protection is not established.

Secretions from normal adults contain relatively little IgM compared with infants and only traces of IgE and IgD. Secretory antibodies are of relatively short duration and are thus of less importance for older children and adults than serum antibodies.

3.7 TRANSPLACENTAL ANTIBODIES

There are many reports of transplacental transfer of antibodies to *b. pertussis* (Cohen & Scandron, 1943; Kendrick *et al.*, 1945; Adams *et al.*, 1947; Miller *et al.*, 1949; Goerke *et al.*, 1958; Marley *et al.*, 1985). Preston (1977) found that the pertussis agglutinin titres of infant sera were directly related to titres in mothers' sera. Burstyn *et al.* (1983) studied the effect of vaccination during the first week of life on the antibody response to PT. The ability of the infants to respond with anti-PT IgG antibody was inversely correlated with the cord anti-PT titre, although this had no influence on the production of IgM anti-PT antibody (IgM does not cross the placenta). Thus, the vaccination of infants too early in life may result in depressed anti-PT IgG antibody responses due to the well-known inhibitory effect of pre-existing antibody. Further evaluation of this phenomenon is required.

3.8 COLOSTRAL ANTIBODIES

Oda *et al.* (1983) discovered that suckling mice were better protected against aerosol *B. pertussis* challenge if they received colostral immunity than if they had obtained transplacental immunity. Similar results were obtained whether the mother mice were vaccinated with acellular or whole-cell vaccine. Transmission of agglutinins through human colostrum were reported (Oda *et al.*, 1983) suggesting that colostral anti-pertussis antibodies may be important in protection of the newborn during the first months of life.

3.9 CELL-MEDIATED IMMUNITY IN PERTUSSIS

The demonstration of a cell-mediated immune response to PT (Fish *et al.*, 1984) suggests a possible role of cell-mediated immunity (CMI) in protection from pertussis disease. Knowledge of CMI in pertussis may be necessary to establish criteria for the evaluation of acellular pertussis vaccines since the absence of detectable serological responses after vaccination may

not necessarily mean that protection has been denied.

3.10 SEROLOGIC RESPONSES TO PERTUSSIS INFECTION

During clinical pertussis serum IgG, IgM and IgA antibodies to *B. pertussis* increase although there may not be a coincidental increase in all three isotypes (Viljanen *et al.*, 1982; Burstyn *et al.*, 1983; Ruuskanen *et al.*, 1980; Zackrisson *et al.*, 1986). The production of high concentrations of IgG to PT, the usual appearance of IgA-FHA, modest agglutinin and modest IgG-FHA responses have been reported in several studies (Long *et al.*, 1990a, Granström 1982, 1988) and is presumptive of recent infection.

IgG production to both PT and FHA is stimulated during the invasive or toxic stage of the disease, titres rise both in those with clinical pertussis and in those who have been exposed to the disease but have no overt clinical symptoms (Wassilak *et al.*, 1983). IgG-PT titres following infection are higher and longer lasting than after vaccination (Sato & Sato, 1984; Linnemann, 1979).

IgM antibodies exist transiently in the earlier phase of the immune response, are indicative of recent exposure to antigen and may be observed in both symptomatic and asymptomatic individuals (Nagel *et al.*, 1985; Ruuskanen *et al.*, 1980; Macaulay, 1981). IgM titres are of little diagnostic value (Granström *et al.*, 1988).

The local production of secretory IgA antibodies in nasopharyngeal secretions during the initial infection stage of the disease may play an important role in inhibiting the colonisation of the respiratory mucosa by *B. pertussis* (Pittman, 1976). Significant increases in IgA-FHA during pertussis have been reported in several studies (Baraff *et al.*, 1983; Burstyn *et al.*, 1983; Wassilak *et al.*, 1983, Granström *et al.*, 1982,1988) and inversely correlated with the

persistence of infection in animal models (Ashworth *et al.*, 1982). IgA titres seldom rise above background levels in those without symptoms of pertussis (Goodman *et al.*, 1981). The role of secretory IgA in providing long-lasting protection against pertussis infection and the extent to which such protection occurs at mucosal sites distant from the site of antigen exposure is not known. IgA responses to FHA and PT may be used to distinguish patients with pertussis from vaccinees. Ashworth *et al.* (1989) found a serum log 10 titre of IgA to PT > 1.9 to be a useful discriminant of *B. pertussis* infection. IgA to AGG2,3 was found to be equivalent in vaccinees and infected infants.

There is little information on IgE responses in pertussis. Finger & Wirsing von Koenig (1985) detected IgE antibodies by ELISA in only 4/125 children with clinical infection.

Immunity is not necessarily associated with the presence of circulating antibodies since these disappear rapidly after infection. Generally one attack of pertussis is sufficient to confer lasting immunity. There is no evidence that nonspecific immunity is acquired with age. The apparent immunity of older children and adults with no history of the disease probably depends on protection resulting from an atypical or unrecognised infection.

3.11 SEROLOGIC RESPONSE TO VACCINATION

The concentrations and type of antibodies to *B. pertussis* antigens that correlate with prevention of disease has not yet been established with certainty for any pertussis vaccine preparation. Vaccine-induced immunity, although not totally protective, can interfere with the growth of *B. pertussis* in the nasopharynx and also *in vitro* (PHLS, 1970).

Whole-cell pertussis vaccination is reported to elicit a brisk rise in agglutinating antibodies and in IgG-FHA; a moderate rise in IgG-PT, a little if any IgA production (Long *et al.*, 1990b). PT and

FHA antibody levels obtained after both acellular and whole-cell pertussis vaccination were found to be comparable to those in convalescents from the disease (Sato & Sato, 1984).

IgG antibodies found in infant sera prior to vaccination are usually passively transferred maternally-derived antibody. The immune response to pertussis vaccination commences with slight IgM synthesis and is followed by a larger output of IgG after a few days (Ruuskanen *et al.*, 1980; Macaulay, 1981; Wassilak *et al.*, 1983).

In general, IgG and IgM seroconversion rates and titres increase with successive doses of vaccine and are higher with increasing age of vaccination, presumably in the case of IgG, because of 'blocking' by maternal antibody in a proportion of younger infants. IgA titres seldom rise above background levels (Goodman *et al.*, 1981; Nagel & Poot-Scholtens, 1983; Burstyn *et al.*, 1983). Specific IgE antibodies to *B. pertussis* have been detected in infants following administration of whole-cell vaccine (Haus & Weinberg, 1988).

Vaccine-induced immunity to pertussis appears to be of relatively short duration, waning after 2-5 years, as reviewed by Trollfors (1984). A decrease in protection relates to the interval since vaccination (Lambert, 1965; Zoffmann, 1982).

Pertussis infection has been reported to occur frequently in vaccinated children through casual exposure to infected individuals in the community. In general the number of doses of vaccine received is proportional to the mildness of disease signs and symptoms (Broome, EPI 1984). One-third to two-thirds of pertussis infection in vaccinated individuals is estimated to be subclinical (Long *et al.*, 1990a).

3.12 MEASUREMENT OF *B. pertussis* ANTIBODIES

3.12.1 ELISA

ELISA is the method of choice for measurement of pertussis antibodies and may also be used to quantitate *B. pertussis* antigens (Ashworth *et al.*, 1983; Baraff *et al.*, 1983; Burstyn *et al.*, 1983, Granström *et al.*, 1982a,b). The technique is both sensitive and specific and allows the different immunoglobulin classes to be quantitated separately in small amounts of sera.

A variety of pertussis antigens have been used in the assay, eg. whole bacteria (Finger & Wirsing von Koenig, 1985), sonicate or extract of bacteria (Lawrence & Paton, 1987; Nagel & Poot-Scholtens, 1983; Viljanen *et al.*, 1982) and purified preparations of different antigens (Sato & Sato, 1985; Winsnes *et al.*, 1984).

The use of ELISA in the serologic diagnosis of pertussis infection has been described in several studies. In 20 different studies in which ELISA techniques were used for the diagnosis of pertussis, the sensitivity varied from 25% to 100% and the specificity from 15% to 100% (Onorato & Wassilak, 1987).The combination of IgG to FHA and PT and of IgA to FHA in diagnosis was found to reach almost 100% sensitivity (Granström *et al.*, 1988). The IgM and IgA anti-pertussis ELISA is considered a valuable tool in the diagnosis of infection, particularly of the atypical form which is mostly culture negative (Kendrick *et al.*, 1969; PHLS, 1970; Viljanen *et al.*, 1982; Conway *et al.*, 1988). An advantage of analysing IgM and IgA antibodies is that they are not maternally derived. Furthermore, IgA antibodies are produced only in response to infection and not to vaccination. Serum anti-PT IgA antibody (Burstyn *et al.*, 1983; Winsnes *et al.*, 1985) and secretory IgA antibody in nasopharyngeal secretions (Goodman *et al.*, 1981) may therefore be used to differentiate between recent infection and vaccination.

ELISA does not require paired sera and its sensitivity is at least 10-100 times higher than bacterial agglutination and complement fixation (Carlsson *et al.*, 1972; Mertsola *et al.*, 1983).

Pre-coating of microtitre plates with fetuin which binds with high affinity to PT has been shown to increase sensitivity and specificity of ELISA for the detection of PT antibodies (Zackrisson *et al.*, 1986).

Disadvantages of the technique are the less reliable antibody response in young infants (43% of culture confirmed cases had negative serologic results (Conway *et al.*, 1988)); and the influence of early antibiotic treatment on the sensitivity of ELISA as a diagnostic tool. Furthermore, ELISA for IgA and IgM FHA antibodies to *B. pertussis* also measures antibody responses to *B. parapertussis*, probably because of the structural similarity or identity of their fimbrial antigens (Granström *et al.*, 1982; Viljanen *et al.*, 1982).

Base-line ELISA titres probably vary with age, vaccination history and the epidemiologic patterns of the region. Comparisons of the results of ELISA as done in different laboratories have been hampered by the lack of standardisation of reference sera, test protocols and the methods of calculating results.

3.12.2 AGGLUTINATION TEST

The agglutination procedure is simple and inexpensive and historically has been the most widely used assay for measurement of immune responses to pertussis. It has been a useful measure of immunity following whole-cell pertussis vaccination. Agglutination tests are of value in epidemiological studies to evaluate immunity to *B. pertussis* in a population, but are of limited use in the evaluation of individual clinical cases. Agglutinating antibody tests detected only 14% of pertussis cases in a recent outbreak in the United States (Fisher *et al.*, 1989).

A variety of methods for performing agglutination tests on pertussis antisera have been described (Evans & Perkins, 1953; Kendrick, 1933; Macaulay, 1979; Miller & Silverberg, 1939; Wilkins *et al.*, 1979). Comparisons of results obtained by different test methods are difficult

because diagnostic antigens differ, as do test methods, incubation times and end-point determinations. A positive agglutination test result requires the presence of only one of the three major agglutin types of *B. pertussis* rather than all three.

In the absence of recent vaccination, a significant increase in antibody titre (≥4-fold) is supportive, but not diagnostic of pertussis infection. The absence of agglutinating antibodies in serum does not exclude diagnosis. The test has limited usefulness in diagnosis, mainly because of the variable but persistent titres present in unvaccinated, unexposed people. False-positive test results are rare, but the sensitivity of the test is poor. The most common reason for the failure to demonstrate a titre increase in a culture-confirmed case of pertussis is the late collection of acute-phase serum sample.

3.12.3 NEUTRALISATION TEST

A diagnostic procedure for anti-PT antibodies is the neutralisation test (NT) performed in microplate cultures of Chinese Hamster Ovary (CHO) cells (Hewlett *et al.*, 1983a; Granström *et al.*, 1988; Gillenius *et al.*, 1985).

In the presence of PT, CHO cells undergo a characteristic clumping by catalysing ADP-ribosylation of a 41 kDa membrane protein (Burns *et al.*, 1987) which is prevented by sufficient anti-PT. Paired serum samples as well as age-matched vaccinated controls are needed to distinguish between antibodies elicited after recent and remove infection. The NT detects <10 ng PT/ml but is significantly less sensitive for the diagnosis of disease than the determination of the IgG response to PT by ELISA (Granström *et al.*, 1988). Since the method is laborious, requires tissue culture facilities and involves subjective readings, it is of little diagnostic value.

CHAPTER 4

PATIENTS

4.1 INTRODUCTION

Two groups of patients were recruited for the various studies presented in Chapters 7 to 12.

1. FULL-TERM INFANTS. This group comprised 345 healthy full-term infants who were enrolled at birth for vaccination with either acellular or whole-cell pertussis vaccines.

2. PRE-TERM INFANTS. This group comprised 34 pre-term infants ranging in gestational age from 28-37 weeks. Four infants were 28-29 weeks, 8 were 30-31 weeks, 8 were 32-33 weeks, 10 were 34-35 weeks, and 4 were 36-37 weeks. Infants who were enrolled at birth or soon thereafter for neonatal vaccination with diphtheria-tetanus toxoids followed by conventional whole-cell DTP at 2, 4 and 6 months.

4.2 SELECTION METHOD AND AREA

1. FULL-TERM INFANTS. In the 3 month period from March to May 1988, 345 healthy full-term male and female newborn infants from Kwa Mashu, a poor periurban suburb of Durban, South Africa inhabited exclusively by blacks were enrolled in the study in sequence at birth provided informed consent was obtained from parents or guardians. Figure 4.1 shows the location of Kwa Mashu in the context of South Africa and Figure 4.2 shows indigenous accommodation.

2. PRE-TERM INFANTS. Infants ranging in gestational age from 28-37 weeks admitted to the neonatal nursery at King Edward VIII Hospital, Durban were enrolled in the study prior to discharge, provided informed consent was obtained from parents or guardians, and they resided in 3 periurban townships of Durban - Lamontville, Umlazi and Chesterville.

4.3 INFORMED CONSENT

Prior to enrollment of the infant, parents or guardians of the infant were informed by the author or a nurse in a language which they clearly understood (usually ZULU) about the aims, benefits and possible side-effects of the vaccination trials, and an information leaflet was given to them (Appendix 2). Following this, written consent was obtained.

4.4 INCLUSION CRITERIA

Full-term or pre-term infants -

1. Infants who were in good physical health as assessed by history taking and physical examination.
2. Infants whose parents or guardians agreed to vaccine administration following a detailed explanation and who signed an informed consent.
3. Infants who were expected to be available for the duration of the vaccination trial up to the age of 9 months.

4.5 EXCLUSION CRITERIA

1. Known or suspected impairment to their immune function or those receiving immunosuppressive therapy.
2. Known or suspected progressive underlying neurological disorder including febrile seizures and developmental retardation, or immediate family history that suggests that the child is at increased risk of developing a neurological disorder.
3. Major congenital malformations or serious chronic disorders (eg. Down's syndrome, diabetes, sickle-cell anaemia, etc.).
4. Any chronic drug therapy to be continued during the study period.
5. A child with parents in a high-risk group for developing an immunocompromising disease.

6. Episode of acute febrile illness at the time of vaccination.

7. Simultaneous participation in any other clinical study.

8. Infants' whose parents knew that they would be moving within 1 year.

4.6 CONTRAINDICATIONS TO VACCINATION

1. Acute illness at time of vaccination (axillary temperature $\geq 37.5^{\circ}C$; undiagnosed rash, respiratory tract infection). Dose postponed until infant is well.

2. Severe local or general reaction to a previous dose as defined below -

   a. An extensive area of redness and swelling which becomes indurated and involves most of the antero-lateral surface of the thigh.

   b. Axillary temperature ($39.4^{\circ}C$) within 48 hours of vaccination.

   c. Persistent inconsolable screaming lasting 3 hours or more within 48 hours of vaccination or an unusual high pitched cry occurring within 48 hours of a dose.

   d. Shock-like episode with cyanosis, pallor or limpness within 48 hours of vaccination.

   e. Generalised allergic reaction (anaphylaxis) within 48 hours of vaccination - bronchospasm, laryngeal oedema, cyanosis, circulatory collapse, prolonged unresponsiveness.

   NOTE Any infant who develops fits or other neurological disorder including encephalopathy at any time during the vaccination course should receive no further pertussis vaccine. A brief episode of twitching of isolated groups of muscles would not be considered a fit unless accompanied by eye-rolling or unresponsiveness.

4.7 WITHDRAWALS

Parents/guardians were free to withdraw their infants from the study for any reason at any time. Subjects would be withdrawn from the study by the investigator in case of a serious side-effect

or suspected health hazard. In all cases, the reason for withdrawal was recorded on the case report form by the investigator. Drop-outs were not replaced.

4.8 RECORDS OF POST-VACCINATION EVENTS

A 14-day follow-up of the clinical signs and symptoms after each vaccination was recorded by parents or guardians of both full-term and pre-term infants on a specially designed illustrated record sheet (Appendix 1). Detailed instructions pertaining to understanding of the record sheet, especially with regard to neurologic events were conveyed in the native language (Zulu) by a registered nurse. Information pertaining to the vaccine trial as well as contact addresses and telephone numbers in Zulu and English were attached to each subject's vaccination record chart (Appendix 2,3).

The following post-vaccination symptoms were solicited -

1. Local reactions at the injection site (swelling, induration, redness).
2. Systemic reactions (clinical impression of fever, appetite changes, excessive crying, fretfulness, hypotonic-hyporesponsive episodes, convulsions or other neurologic events).
3. Quantitative measurements of fever and diameters of induration and swelling were not made, as reported in other studies of vaccine reactogenicity, because of inadequate facilities and limited educational background of parents.

The parents or guardians were instructed to contact the investigator or study nurse immediately in case of one of the following -

1. Suspicion of whooping cough disease lasting 7 days or more, with or without associated whoop or vomiting. These diagnostic criteria are the same as those used by

the WHO in clinical trials of acellular pertussis vaccines in Sweden (*Ad hoc* Group for the Study of pertussis vaccines, 1988).

2. Neurological reactions such as convulsions, collapse and/or unexplained loss of consciousness, infantile spasms.
3. General allergic reactions.
4. Any serious reaction of an unforeseen nature where the child's life is endangered.
5. Several local reaction as described above.
6. Any other sign or symptom that caused the child's attendants to seek medical assistance.

4.9 CLINICAL FOLLOW-UP

All infants underwent physical examination by a paediatrician at birth and at every clinic visit (2, 4, 6 and 9 months of age). Intercurrent illnesses were monitored through monthly home-visits by a study nurse, with special attention being paid to nutritional state, common childhood infections, and whooping cough symptoms. In addition, information pertaining to gestational age and APGAR score were recorded in pre-term infants.

4.10 COLLECTION OF SOCIO-DEMOGRAPHIC DATA

Mothers were interviewed by a nurse or the investigator prior to discharge from the post-natal ward. Interviews were conducted in the mothers' language of choice. The following data were recorded for each infant -

1. Mothers' educational level (number of years).
2. Time-interval (years) from previous live birth (birth interval).
3. Attendance at vaccination clinic until 9 months of age (number of visits). (The number of visits required for completion of primary vaccination was 4).

4. Gender (infant).

4.11 ASSESSMENT OF NUTRITIONAL STATUS

The following data were recorded for each infant -

1. Anthropometric indices of nutritional status in mother prior to discharge from the post-natal ward (height, weight, mass index)

   Mass index = $\frac{\text{weight}}{\text{height}}$

2. Anthropometric indices of nutritional status in infant at birth (length, weight, head circumference) (OFC) and at 2, 4, 6 and 9 months of age (weight and length).
3. Clinical features of protein-energy malnutrition, vitamin and trace-element deficiencies.

A variety of research techniques may be used in assessing nutritional status. These include determinations of biochemical parameters in various tissues, clinical examination of subjects, and nutritional anthropometric indices.

Anthropometric indices are extensively employed and are generally considered highly sensitive indicators of nutritional status. A WHO Expert Committee on Nutritional Surveillance (WHO Technical Report Series, 1976) has recommended the use of the indices of length-for-age and weight-for-age as primary indicators of nutritional status in children. Cut-off points between acceptable nutrition and malnutrition may be expressed as 'percentiles' or 'percentage of the median'.

Percentiles: The number of the percentile represents a position out of 100. The fiftieth percentile represents the midpoint of the population; half the children will be above and half below this value.

Percentage of the median: This is calculated by first identifying the median value for the reference population. This value is taken to represent 100%. Absolute values at different percentage units from the median are calculated.

Reference population (full-term infants): The National Centre for Health Statistics (NCHS) Reference Population Compiled from Studies at the Fels Research Institute, Yellow Springs, Ohio, USA; and selected from different economic and ethnic groups in the USA was used as a standard for full-term infants.

The data from this reference population are available for both sexes as centile curves and tables of weight-for-age, height-for-age, head circumference and weight-for length (National Centre for Health Statistics, 1976) (Appendix 4,5)

Weight-for-age: Weight was measured on a beam balance scale with the child unclothed to the nearest 10g. Weight deficiency appears to be the best indicator of the prevalence of PEM in children of all age groups. Comparison of weight-for-age values with regional standards at corresponding ages determines the current nutritional status of the child but does not distinguish between acute and chronic malnutrition.

Length-for-age: Measurement of supine length was made with a standard measuring board. An investigator positioned the child's head firmly against the fixed head-board and moved the footboard into firm contact with the soles of the feet whilst ensuring full extension of the lower extremities. Length was measured to the nearest tenth of a millimetre.

The extent of length deficit in relation to age, as compared to standards is regarded as a measure of the duration of malnutrition. A given deficit in length at an early age represents a short period of growth failure assesses past and chronic PEM.

Head circumference (OFC):

Head circumference represents the maximum measurement around the head in the horizontal plane. It is measured with a non-stretchable tape at the maximum point of occipital protuberance posteriorly and above the eyebrow anteriorly. It may be influenced by hair growth, ie. 1 mm of scalp hair thickness will increase the circumference by about 6 mm.

Method of classification: Nutritional status of subjects was assessed at each clinic visit by anthropometric indices of length- and weight-for-age; and clinical features of PEM, vitamin or trace-element deficiencies. Data for an individual infant (sex, age, length, and weight) were used to place the infant in the appropriate percentile. Infants with a length-for-age below the 3rd percentile were considered underweight (WHO Classification System). Infants with a length-for-age less than 90% of the median were considered stunted (CDC Classification System). Infants with a weight- and length-for-age less than the 5th percentile were considered wasted (Waterlow *et al.*, 1977).

Low-birth-weight (LBW) was defined as a birth weight less than 2500 g.

Small-for-gestational-age (SGA) was defined as a birth weight or weight-for-length on or below the 10th percentile curve (Lubchenco *et al.*, 1966).

Pre-term infants: ie. those born with a gestational age <37 weeks. Gestational age was assessed according to the Dubowitz score, derived from neurological plus external criteria (Dubowitz *et al.*, 1970). Nutritional status was assessed according to a combined intrauterine-neonatal growth chart for height, weight and head circumference (Lubchenco, 1966). This is shown in Appendix 6.

Mass index: This is the ratio of weight/height. It is independent of age but correlates with nutritional status and is sensitive to changes thereof. Weight-for-height can conceal chronic

malnutrition because there is a failure in both mass and height with little change in the ratio (Dugdale, 1971).

4.12 RESULTS

4.12.1 SEX RATIO

Full-term infants: The male:female ratio was 174:171 ie. 1.02:1.

Pre-term infants: The male:female ratio was 11:23, ie. 0.48:1.

4.12.2 POST-VACCINATION EVENTS

Details of events in full-term infants are given in Chapters 7 and 8, and in pre-term infants in Chapter 10.

4.12.3 CLINICAL RECORD

Full-term infants: Age-related incidence of intercurrent illnesses, infection, and clinical signs thereof in full-term infants from birth to 9 months of age is shown in Table 4.1.

Clinical signs and symptoms of the upper respiratory tract (otitis media, otitis externa, tonsillitis and 'glue ear'); lower respiratory tract (bronchopneumonia); gastro-intestinal tract (diarrhoea, blood in stools, vomiting, colic); and skin infection (scabies, eczema, 'heat' rash, impetigo, seborrhoeac dermatitis, vaseline allergy, abscesses, boils and intertrigo) were recorded. Subclinical pertussis occurred in 10 infants; 1 other infant experienced clinical signs of the disease (Further details are given in Chapter 9).

Cranial and CNS-related signs included febrile convulsions (n=1), large anterior fontanelle (n=3), ossified cephalhaematoma (n=1), abnormal head structure (n=1), 'floppy' infants (n=3); bossing (n=1), and craniotabes (n=2). The age group in which the above signs occurred are

shown in Table 4.2.

Pre-term infants: Clinical signs and symptoms in pre-term infants from birth to 9 months of age are shown in Table 4.3. Upper respiratory tract infections (ear-nose-throat, common cold) were the most commonly occurring (25% of illnesses). Most illnesses were experienced between birth and 2 months of age (44.6% of illnesses). Three infants experienced pertussis infection - 2 between 2 and 4 months and 1 between 4 and 6 months.

4.12.4 SOCIO-DEMOGRAPHIC DATA

Data pertaining to mothers education, birth interval and attendance at vaccination clinic obtained from mothers of full-term infants are presented in Table 4.4.

4.12.5 NUTRITIONAL INDICES

Nutritional indices of full-term infants and their mother's are presented in Tables 4.5 and 4.6. Further details are given in Chapter 11. Nutritional indices of pre-term infants and their mothers are presented in Tables 4.7 and 4.8. Further details are given in Chapter 10.

4.12.6 DROP-OUTS

Full-term infants: Three vaccination unrelated deaths occurred before 2 months of age. All 3 infants had received only BCG and TOPV at birth. Of the 342 remaining subjects, 285 (83%) returned at 2 months of age, 256 (75%) at 4 months, 232 (68%) at 6 months and 198 (58%) at 9 months of age.

Pre-term infants: Thirty-four infants were enrolled at birth, 16 (47%) of whom returned at 2 months, 14 (41%) at 4 months, 11 (32%) at 6 months and 6 (18%) at 9 months.

TABLE 4.1 Age-related incidence of intercurrent illnesses, infections and clinical signs thereof in full-term African infants from birth to 9 months of age*.

ILLNESS/INFECTION	NUMBER OF CASES/AGE GROUP (MONTHS) [%]							
	0<2		≥2<4		≥4<6		≥6<9	
Upper respiratory tract	25	[32.9]	42	[37.3]	49	[38.9]	69	[37.7]
Skin	18	[23.7]	34	[30.1]	32	[25.4]	50	[26.0]
Diarrhoea	3	[3.4]	10	[8.8	12	[9.5]	30	[16.4]
Lower respiratory tract	5	[6.6]	11	[9.7]	11	[8.7]	13	[7.1]
Pyrexia of unknown origin	2	[2.6]	4	[3.5]	4	[3.3]	10	[5.0]
Eyes	5	[6.6]	5	[4.4]	4	[2.7]	2	[1.0]
Measles	-		-		-		5	[2.7]
Chicken pox	-		1	[0.9]	-		3	[1.6]
Viral meningitis	-		-		2	[1.6]	1	[0.5]
Hepatosplenomegaly	3	[3.9]	4	[3.5]	7	[5.6]	1	[0.5]
Jaundice	5	[6.6]	-		2	[1.6]	-	
Ventricular septal defect	1	[1.4]	-		-		-	
CNS related	6	[7.9]	1	[0.9]	5	[2.7]	1	[0.5]
Anaemia	3	[3.9]	1	[0.9]	-		-	
Oral herpes	-		-		-		2	[1.0]
TOTAL	78		113		126		183	

* Excluding pertussis infection.

TABLE 4.2 Age of occurrence of cranial or central nervous system-related symptoms in full-term infants.

SYMPTOM	NUMBER OF CASES/AGE GROUP (months)			
	0-2	2-4	4-6	6-9
Febrile convulsions	1	-	-	-
Large anterior fontanelle	-	-	3	-
Ossified cephalhaematoma	1	-	-	-
Abnormal head structure	1	-	-	-
'Floppy' infant	1	-	1	1
Bossing	-	1	-	-
Craniotabes	1	1	-	-

TABLE 4.3 Clinical signs and symptoms in pre-term infants from birth to 9 months of age*.

SYMPTOM	AGE GROUP				
	01≤2	>2≤4	>4≤6	>6≤9	TOTAL
Skin	1	3	1	1	6
Upper respiratory tract	1	4	5	4	14
Lower respiratory tract	2	-	-	-	2
Gastrointestinal tract	-	1	1	1	3
Hepatosplenomegaly	-	-	1	1	2
Measles	-	-	-	1	1
Anaemia	-	1	-	1	2
Fever	2	-	-	-	2
Umbilical hernia	1	-	1	-	2
Fracture	-	-	-	1	1
Abscess	-	-	-	1	1
Ejection systolic murmur	-	-	1	-	1
Cor-pulmonale	-	1	-	-	1
Jaundice	3	-	-	-	3
Bilateral intraventricular haemorrhage	1	-	-	-	1
Hyaline membrane disease	6	-	-	-	6
Respiratory distress	8	-	-	-	8
TOTAL	25	10	10	11	56

* Excluding pertussis infection.

TABLE 4.4 Socio-demographic data (full-term infants).

	MEAN	SD	N	RANGE
Mothers' education (yrs)*	8.7	2.3	329	0-15
No of clinic visits**	2.7	1.5	345	0-4
Birth interval (yrs)	4	2.4	248	1-13

* Completion of secondary education = 12 years.
** 4 visits were requested from 0-9 months of age.

TABLE 4.5 Nutritional indices in mothers' of full-term infants.

	MEAN	SD*	N	RANGE
Height (cm)	154.8	6.8	345	139-175
Weight (kg)	67.9	11.7	344	46-102
Mass index**	0.44	1.72	344	0.33-0.58

* Standard deviation

** $\frac{\text{Weight}}{\text{Height}}$ ratio

TABLE 4.6 Birth weight in 345 full-term African infants.

	BIRTH WEIGHT (kg)
Mean	3.2
SD	0.5
Range	1.72-4.28
N	345

TABLE 4.7 Assessment of nutritional indices in pre-term infants at birth.

NUTRITIONAL INDICES	PERCENTILE <10	10-50	50-90
Weight/age	7	9	18
Length/age*	17	5	7
OFC/age*	5	8	15

* Not available for all infants.

TABLE 4.8 Nutritional indices in 33 mothers of pre-term infants.

	MEAN	SD	RANGE
Height (cm)	154.6	7.1	145-171
Weight (kg)	66.5	8.3	51-90
Mass index (weight/height)			0.35-0.53

KWAZULU IN THE CONTEXT OF SOUTH AFRICA

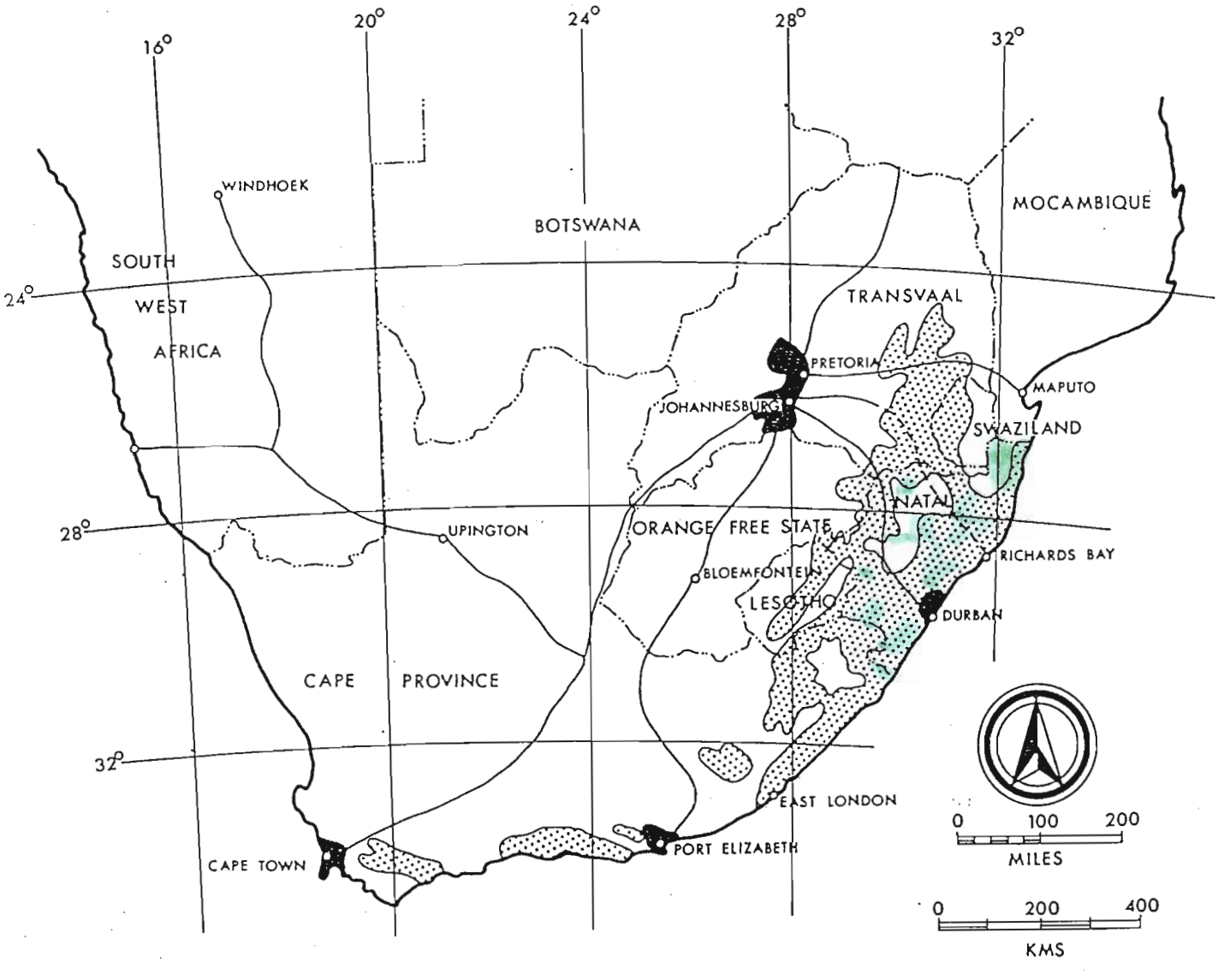


Figure 4.1 Kwa Zulu in the context of South Africa.

FIGURE 4.2 Kwa Mashu polyclinic, Kwa Zulu.

FIGURE 4.3 Residential area of full-term infants (Kwa Mashu).

CHAPTER 5

METHODS

5.1 INTRODUCTION

Serologic assays for the detection of antibodies to specific *B. pertussis* antigens and to diphtheria toxoid were carried out by the author at the Centre for Applied Microbiological Research, Public Health Laboratory Services, Porton, UK. ELISA for the detection of antibodies to tetanus toxoid was carried out at the Department of Paediatrics and Child Health, University of Natal.

5.1.1 *Bordetella pertussis* SEROLOGY

Paired pre- and post-vaccination serum samples were tested for the presence of antibodies against pertussis toxin (PT), filamentous haemagglutinin (FHA) and fimbrial agglutinogens 2 and 3 (AGG2,3).

i. Antibodies against PT: Three methods are described: ELISA, a neutralisation of the toxic effect of PT in an *in vitro* assay using cell cultures, and immunoblotting. In the ELISA and immunoblotting, antibodies against most of the epitopes of PT are detected, whereas in the neutralisation, only antitoxin antibodies are measured.

a. *ELISA.* This assay is a sandwich method in which PT is bound to the solid phase (Rutter *et al.*, 1988). The titre of each serum sample (expressed in ELISA U/ml) is calibrated against a Japanese reference serum by means of parallel line assays.

b. *Neutralisation assay.* The toxic effect of PT and its neutralisation by antibodies are evaluated on Chinese hamster ovary cells (CHO) as described elsewhere (Gillenius *et al.*, 1985). Serial 2-fold dilutions of each serum titre were made. The end-titre was the reciprocal of the last dilution that completely inhibited the toxicity of 4 cytopathogenic units of PT on CHO cells.

c. *Immunoblotting.* This was used to evaluate the specificity of the antibody responses to vaccination and to confirm the ELISA data.

ii. Antibodies against FHA: These antibodies are measured by an ELISA assay. This assay is a sandwich method in which FHA is bound to the solid phase. The titre of each serum sample (expressed in ELISA U/ml) is calculated against Japanese reference serum by means of parallel line assays.

iii. Antibodies against AGG2,3: These antibodies are measured by an ELISA assay. This assay is a sandwich method in which the antigen AGG2,3 is bound to the solid phase. The titre of each serum sample (expressed in ELISA U/ml) is calculated using the parallel line method with Japanese reference serum calibrated in arbitrary IgG ELISA units.

5.1.2 VACCINE QUALITY CONTROL

i. *Neutralisation assay*. Vaccine toxicity was measured by the neutralisation test using microplate tissue cultures of Chinese hamster ovary cells (Gillenius *et al*., 1985).

ii. *Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.* Purity of PT and FHA antigens in acellular vaccine were qualitatively assessed by SDS-PAGE and immunoblotting.

5.1.3 DIPHTHERIA AND TETANUS SEROLOGY

These antibodies were measured with an ELISA technique.

i. *Antibodies agalnst diphtheria toxoid*. This assay is a sandwich method in which diphtheria toxoid is bound to the solid phase (Camargo *et al*., 1984). The titres are expressed as IU/ml with the human reference serum obtained from the National Institute of Biological Standards and Controls (NIBSC), UK. The cut-off of the test was 0.1 IU/ml.

ii. *Antibodies against tetanus toxoid*. The assay is a sandwich method in which tetanus

toxoid is bound to the solid phase. Titres were expressed as IU/ml with reference serum provided by the Natal Blood Transfusion Service, South Africa. The cut-off of the test was 0.1 IU/ml.

5.2 SERUM SAMPLES

5.2.1 SAMPLE RECEIPT, RECORDING AND PREPARATION

Blood samples were obtained by venipuncture from cord blood and mothers' blood at birth and again at 2, 4, 6 and 9 months of age. Blood samples in plain-glass stoppered tubes were left to stand at room temperature for 3-4 hours, centrifuged and the serum aliquoted into 0.5 ml polystyrene tubes for storage at -20°C.

The period of storage prior to assay varied between two to ten months. Storage for such periods had been shown not to affect the quantification of antibodies (Miller *et al.*, 1991). Indeed, sera kept at 4°C and at -20°C over a 3 yr period showed only a 3% difference in antibody activity. Sera were heat-treated (56°C for 30 min) before screening for pertussis antibodies.

5.2.2 SAMPLE GROUPING

Serum samples from each subject were tested as a group, and assayed on one ELISA plate in order to minimise methodological variability.

5.3 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR SERUM IgG AND IgA ANTIBODIES TO PT, FHA AND AGG2,3

5.3.1 ANTIGEN COATING OF MICROTITRE PLATES

Introduction: Microtitre plates specifically coated with purified PT, FHA or AGG2,3 antigen preparations by a direct binding of the antigen to the plate, were used to compare the antibody activity of the test sera with that of the reference sera. The optimal concentration of antigen for

coating of microtitre plates was determined in advance of their use in the assays by chequerboard titration (Appendix 12).

Antigens: The three test antigen preparations, FHA, PT and AGG 2,3 were supplied freeze-dried for storage at 4 °C. The freeze-dried antigens were reconstituted in 50% (V/V) glycerol in water and stored thereafter at -20 °C. Purified pertussis PT and FHA was supplied by the BIKEN-KANONJI Institute (Japan). Each vial contained 200 μg protein nitrogen of purified PT or FHA. Properties of the antigens are shown in Figure 5.1. Purified Agglutinogens 2,3 were supplied by the Biologics Division PHLS, CAMR, Porton Down, UK, containing 500 μg/ml protein nitrogen. This was prepared from a serotype 1,2,3 strain and contained AGG2,3 in a ratio of 6:4. Details of preparation are given in Appendix 10. Vaccine preparations could not be used to coat plates since these act as immunogens but not as antigens.

Materials: Details of reagent preparation for use in ELISA are given in Appendix 11.

1. Twelve-channel adjustable volume pipette covering the range 25-200 μl.
2. Automatic ELISA washer (TITERTEK(R), Flow Laboratories).
3. Adjustable volume single channel pipettes (Finnpipette) covering the ranges 5-50, 50-200 and 100-1000 μl.
4. Coating buffer: 0.05M carbonate-bicarbonate buffer pH 9.5.
5. Antigens: The antigens were diluted in coating buffer immediately prior to use. The concentrations of antigens used (μg/ml) were as follows :

	FHA	PT	AGG2,3
IgG	0.5	2.0	2.0
IgA	1.0	2.0	2.0

6. Microtitre plates: Flat-bottomed 96-well polystyrene plates (NUNC IMMUNOPLATE I,

Catalogue No 4-39454); Gibco Bio-Cult, Paisley, Scotland).

7. Wash solution: 0.01M PBS pH 7.4 containing 0.1% Tween 20 to prevent nonspecific binding.

Method:

1. Optimal concentrations or 5 μg of antigen (FHA, PT or fimbrial agglutinogen) per ml was diluted in coating buffer and 100 μl placed in wells of polystyrene microtitre plates using a 12-channel pipette. Plates were vibrated for 20-30 seconds by a micromixer, covered with plastic lids and adsorption of the antigen onto the plates was allowed to proceed overnight at 28°C.
2. Plates were washed 3 times by adding and aspirating the wash solution, then blocked with 100 μl volumes of PBS containing 1% newborn calf serum for 1 hour at 28°C.

5.3.2 ASSAY PROCEDURE

Introduction: This assay is a sandwich method in which the antigen is adsorbed onto a polystyrene microtitre plate. The method was developed at CAMR by Rutter *et al.*, 1988 and modified for use with the above reference sera and antigen preparations. IgG and IgA ELISA have been confirmed to be more sensitive than agglutination tests for evaluation of immune responses to pertussis (Lawrence & Paton, 1989; Steketee *et al.*, 1988).

Materials:

1. Assay plates: NUNC 96-well polystyrene microtitre plate immunoplate I, catalogue number 4-39454, coated with the appropriate antigen as described above.
2. Dilution plates: Sterilin or any other cheap 96-well plate.
3. Reagent trays: Large size disposable weighing dishes.
4. Wash solution: 0.01 M PBS pH 7.4 containing 0.1% Tween 20 to prevent nonspecific binding.
5. Serum diluent: Wash solution containing 10% newborn calf serum as a further blocking

agent.

6. Conjugates: Horseradish peroxidase conjugates of affinity-purified goat IgG antibodies to human IgG or IgA (purchased from Northeast Biomedical Laboratories, Uxbridge, UK). Once reconstituted according to the manufacturers' instructions the conjugates were stored at 4°C. The appropriate working dilution of each batch of conjugate was determined in advance of their use in the assays (Appendix 13). 1:5,000 dilution in ELISA diluent was carried out immediately prior to use.

7. *Pertussis reference serum.* Anti-PT, anti-FHA Human IgG (Lot 10). Supplied by BIKEN-KANONJI (Osaka, Japan) as a freeze-dried product containing 250 ELISA U/ml of PT antibody and 400 ELISA U/ml of FHA antibody. Reconstituted to 1 ml in distilled water and diluted 200 times in washing buffer before use in assays. This reference serum was assayed versus the current standard used at CAMR and assigned a unitage of 620 ELISA U/ml anti-AGG2,3 antibody. CAMR anti-human IgA standard was used for IgA assays.

8. Substrate: 3,3',5,5'-Tetramethyl benzidine (TMB), Aldrich Chemical Co, Catalogue Number 86033-6) was made immediately before use as follows: A solution of TMB at 10 mg/ml dimethyl sulphoxide was prepared and 0.1 ml of this solution was added per 10 ml 0.05M acetate buffer pH 6.0 with constant mixing (the acetate buffer was prepared as a 0.5M stock and diluted with water). Care should be taken that the vessel used for mixing is ultra clean and that the buffer is > 20°C, otherwise the TMB may come out of solution. Finally, 2 µl 100 vol (ie. 30%) hydrogen peroxide was added per 10 ml of the buffered substrate.

9. Sulphuric acid: A 20-fold dilution of concentrated sulphuric acid was made by adding the acid dropwise to stirred distilled water.

10. Adjustable volume single channel pipettes (Finnpipette) covering the ranges 5-50, 50-200 and 100-1,000 µl with stocks of appropriate tips.

11. Twelve-channel adjustable volume pipette(s) covering the range 25-200 µl.

12. Automatic ELISA washer (Flow Laboratories).

13. Programmable automatic diluter/dispenser (Probus "Quatro").

14. Orbital plate shaker (Flow Laboratories).

15. Plate reader with 450 nm filter and computer interface (Flow Laboratories). Computer, BBC model B with double disc drive dedicated to running the plate reader.

Method: All stages were performed at room temperature and plates were fitted with lids or stacked whenever possible.

Assay layout: Any group of sera was tested for antibodies of one Immunoglobulin class against the three test antigen preparations within the same test (ie. on the same day). To do this a single 8-well 3-fold dilution series was made for each serum and aliquots from this series were transferred to three assay plates, each coated with a different antigen. The reference serum at its working dilution was run twice on each plate, leaving room for one blank row and nine titrations of test sera.

Dilution procedure for serum samples: Diluent was distributed into uncoated dilution plates using a 12- channel adjustable pipette; 255 μl into the first well of each dilution series and 180 μl into each of the other seven wells. A volume of 15 μl of reference serum, or test serum, was added to the first well using a Finnpipette, transferring the tips in order to the 12-channel pipette set at 90 μl. When all the sera was added to a plate, the contents of the serum-containing wells were mixed thoroughly using the correctly orientated 12-channel pipette and 90 μl was transferred from well 1 to well 2 of each series. After mixing again, the three-fold dilution series was continued through all 8 wells. A summary of dilution protocol is shown below (volumes are expressed in μl).

TRIAL SERUM (DILUTION)	SAMPLE VOLUME	VOLUME OF DILUENT		SERIAL TRANSFER
		WELL 1	WELLS 2-8	
3-fold	15	225	180	90

Assay:

1. Using the 12-channel pipette and working from high to low, a volume of 50 µl was transferred from each well of the dilution series to the corresponding well of an antigen-coated plate.

   50 µl of diluent was then added to each well. The plates were covered and placed on the shaker for two hours at room temperature.

2. The plates were washed (a washing cycle involved emptying the wells, then filling and emptying them twice) and 100 µl of diluted conjugate was added to all wells. The plates were covered and shaken for two hours.

3. The plates were washed again. Substrate (100 µl/well) was added and plates were covered and shaken until blue colour development was satisfactory (10-20 minutes).

4. The colorimetric reaction was terminated when necessary by the addition of 30 µl diluted sulphuric acid to each well; the colour turned from blue to yellow.

5. The base of each plate was wiped, the lid removed and having blanked the plate reader on an empty assay plate, the test plates were read at 450 nm. By comparing the absorbance obtained with the test serum dilutions with those of the reference serum, the antibody activity (titre) of the test serum was calculated with a parallel line assay and expressed in ELISA IU/ml.

Within an assay, all the plates for antibodies to one antigen were read in sequence and the data stored on floppy discs.

5.4 ELISA FOR ANTIBODIES TO DIPHTHERIA TOXOID

Materials:

1. *Antigen:* Diphtheria toxoid 3550 LF/ml. Supplied by the National Institute of Bacteriological Standards and Controls (NIBSC), UK. MW 63,000.

2. *Reference serum:*
   a. Human Diphtheria Antitoxin standard serum obtained from NIBSC, UK, containing 0.8 IU/ml antitoxin was used for reference purposes.
   b. Pooled serum of 10 recipients of 3 doses of Japanese acellular pertussis vaccine and 10 recipients of 3 doses of South African whole-cell pertussis vaccine. Unitage of this serum was calibrated against that of UK standard serum and was determined as 3.58 ± 1.19 IU/ml.
3. *Conjugate:* Horse-radish peroxidase conjugate of affinity purified goat anti-human IgG (North East Biomedical Laboratories).
4. *Blocking buffer:* 20% newborn calf serum.
5. *Substrate buffer:* TMB.
6. *Stopping solution:* (sulphuric acid).
7. *Dilution plates:* Sterilin or any other cheap 96-well plate.
8. *Microtitre plates:* NUNC 96-well polystyrene plate (Immunoplate I, Cat No 4-39454).
9. *Wash solution:* 0.01M PBS pH 7.4 containing 0.1% Tween 20.
10. *Serum diluent:* Wash solution containing 10% newborn calf serum.
11. *Adjustable volume single channel and 12-channel pipettes* (Finnpipette).
12. *Programmable automatic diluter/dispenser* (Probus "Quatro").
13. *Plate reader* with 450 nm filter and computer interface (Flow Laboratories.
14. *Orbital plate shaker.*

5.4.1 OPTIMAL DILUTION OF DIPHTHERIA TOXOID FOR COATING OF MICROTITRE PLATES

A chequerboard format was used to ascertain the optimum dilutions of diphtheria toxoid and

positive reference serum for performing the immunoassay.

Dilution procedure for reference serum:

1. A ten-fold dilution of each reference serum was made with diluent and 60 µl volumes were distributed into each well of the eighth row of the dilution plate (Row H).
2. The contents of the wells were mixed and 120 µl amounts of diluent were then added to each well in the plate.
3. Using a 12-channel adjustable pipette, 60 µl amounts were transferred from well one to well two of each row.
4. After mixing again, the three-fold dilution series was continued through all wells.

Method:

1. Three dilutions of diphtheria toxoid, 1:5,000, 1:7,500 and 1:10,000 were made in coating buffer.
2. 50 µl of each dilution was used to coat wells of a microimmunoplate. The first and last two rows contained only coating buffer.
3. Plates were covered and kept overnight at room temperature then washed and blocked for one hour with 20% newborn calf serum.
4. 100 µl amounts of diluted reference serum were transferred from dilution plate to wells of the test plate as per diagram below :

DIPHTHERIA TOXOID SOLUTION		1:5,000			1:7,500			1:10,000				
ROW	1	2	3	4	5	6	7	8	9	10	11	12
Reference Serum	Blank	Pool	Pool	UK	Pool	Pool	UK	Pool	Pool	UK	Blank	Blank
	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

5. Plates were shaken at room temperature for 2 hours then washed and 50 μl/well of 1:10,000 conjugate was added.
6. Plates were shaken again for two hours at room temperature then washed.
7. Substrate solution (100 μl/well) was added and plates were shaken until optimal blue colour developed (10-20 minutes).
8. The reaction was stopped by the addition of 30 μl sulphuric acid to each well.

5.4.2 ASSAY OF IgG-DIPHTHERIA ANTITOXIN BY ELISA

The ELISA for the measurement of IgG antibodies to diphtheria toxoid was modified from the method of Melville-Smith *et al.* (1983). Purified diphtheria toxoid and human reference serum was obtained from Dr. Keith Redhead, NIBSC, UK. Results were expressed as IU/ml by calibration against this reference serum.

Method:

1. Microtitre plates were coated overnight at 28°C with 375 ng/ml diphtheria toxoid (0.135 LF/ml) diluted 1:5000 in coating buffer.
2. Plates were blocked for 1 hour with 20% newborn calf serum.
3. Plates were washed again and several dilutions of sera were made, 50 μl of which were added to duplicate wells. Buffer was added to all wells (negative control) and standard (positive control) were included in the assay. The plates were covered and shaken for 2 hours at 28°C.
5. The plates were washed again. Substrate (100 μl/well) was added and plates covered and shaken at 28°C until a blue colour developed (10-20 minutes).
6. The colorimetric reaction was terminated by the addition of 30 μl stopping solution to each well.
7. Absorbance of plates was read at 450 nm.

Calculation of results: For determination of IU a standard curve was constructed using reference serum. Antibody levels in test sera were calculated relative to the reference.

5.5 ELISA FOR IgG ANTIBODIES TO TETANUS TOXOID

5.5.1 INTRODUCTION

Antitetanus toxin antibodies in sera were assayed by a sandwich ELISA in which tetanus toxoid as adsorbed onto polystyrene microtitre plates after the method of Melville-Smith *et al.* (1983) with some modifications thereof (Conradie & Mbhele, 1980).

The gold standard for determining antibodies to tetanus toxin is the mouse neutralization test. This test is however far too cumbersome for large scale serological testing. The ELISA has been shown by various workers to give excellent correlation with the mouse neutralization test and to have similar sensitivity (down to 0.01 IU) (Melville-Smith *et al.*, 1983; Cox *et al.*, 1983).

Reagents and microtitre plates coated with purified tetanus toxoid were purchased from the Natal Blood Transfusion Service, Durban, South Africa. All the assays included a reference serum calibrated against mouse protection studies.

5.5.2 MATERIALS AND APPARATUS

1. Polystyrene microtitre plates (DYNATECH M129B) precoated with purified tetanus toxoid at 5 LF/ml (LOT A-760). This concentration was shown to be optimal in preliminary assays.
2. Tetanus negative control: sample diluent.
3. Human anti-tetanus standard positive control sera designated C_1, C_2 (LOT A-185). 32 IU relative to cell culture studies carried out at the National Institute of Virology, South Africa.
4. Conjugate: Sheep anti-human IgG horse-radish peroxidase (1/6000 dilution). Diluted

5 μl/30 ml in TST.

5. Chromogenic substrate: 1.2 mg OPD per ml of substrate buffer to be used within 60 minutes.
6. Stopping solution: Sulphuric acid 3M.
7. Tris Saline Tween (TST) pH 8.0 (0.5 ml Tween 20 (SIGMA P-1379) to 1 litre.
8. Sample diluent (SBTS): 2 ml neutral sheep serum, 2 ml neutral bovine serum and 96 ml TST.
9. Behring ELISA processor.
10. Titertek multi-channel pipette.

5.5.3 METHOD

1. A sealed humid container was placed in a 45°C incubator for at least 30 minutes before commencement of assay.
2. Serial dilutions of the anti-tetanus standard were made to give the following concentrations: 32 (undiluted), 16, 8, 4, 2, 1, 0.5 IU/ml. The standard reference serum used was adult serum with the highest relative tetanus antibody found from screening adult serum samples.
3. Serum samples were diluted 1/500 by mixing 10 μl serum with 4990 μl, sample diluent 50 μl diluted serum (1/500) was added to each well in the microtitre plate. The plate was then incubated at 45°C in a sealed humid chamber and washed twice with TST.
4. 50 μl diluted conjugate was added to each well and the plate was again incubated at 45°C for 1 hour.
5. After washing twice with TST, 50 μl substrate buffer (chromogen) at room temperature was added to each well and then plate incubated at 45°C for half hour.
6. The reaction was stopped by the addition of 100 μl 3M sulphuric acid.
7. Optical densities were measured at 492 nm.

5.5.4 CALCULATION OF RESULTS

For determination of international unitage (IU) a standard curve was constructed using reference serum by the method of Zollinger & Bostego (1981). This is shown in Figure 5.3. Levels of antibody in each serum sample were calculated relative to this reference.

≥5 IU was designated a low titre test (LTT), indicative of vaccination.

≥30 IU was designated a high titre test (HTT), indicative of treatment.

5.6 ELISA DATA PLOTTING AND CALCULATION OF RESULTS

5.6.1 DATA PLOTTING (PT, FHA, AGG2,3, DIPHTHERIA ANTIBODIES)

The computer printout of results was in the form shown in Figure 5.1. These results were also stored on floppy disc and this raw data was formatted by the ELISA software (Written by Dr. C. Shone, CAMR) according to a pre-determined format. Formatted data was stored on the second side of the ELISA data floppy disc. The formats used for serum IgG and IgA antibodies were similar but different in initial dilution and in the dilution series used. Once formatted, the data could be graphed, titres calculated and, by using the titrations of the reference serum run in tracks two and eleven, unitage calculated. The software was designed to use only one reference plot for calculation of unitage. All the results were processed in the following manner.

Using the software graphing programme the results for each track were plotted by the software. Data for each test sample and the reference serum are plotted as log 10 dilution versus the optical density of 450 mm (Figure 5.2, 5.3). Points which were obviously spurious were rejected by the operator at this time, as were points which did not contribute to the form of the plot. This was done because the programme required at least three points to draw a straight line and at least four points to draw a best-fit curve (either second or third order). Any points which were accepted, ie. not positively rejected, were included in the software calculations to determine the line drawn. Although the form of the plot chosen and the titre can be printed out

this is a slow process, and the titre at the designated end-point were therefore handwritten on the ELISA results printout. The end-point used lines on the most linear portion of the dose response plot. A straight-line plot cannot be used in every case and it is therefore not possible to assign a gradient to every plot. The software was allowed to choose the best-fit curve after pruning.

5.6.2 USE OF THE REFERENCE PREPARATION TO CALCULATE UNITAGE

ELISA antibody units for the test sera were determined relative to the reference sera. For each microtitre plate, in order to compensate for inter-plate and inter-test variation, the log mean of the titres obtained from the two titrations of the reference preparation on each plate were calculated and used to determine the unitage of the specific antibodies. The unitage of the test serum was determined by multiplying its relative titre by the assigned unitage of the reference serum

$$\frac{\text{Titre of test sample}}{\text{Log Mean Titre of Reference Serum}} \times \text{Unitage of Reference Serum}$$

If the lower of the two titres found for the reference preparation on one assay plate was less than 50% of the higher, only the titre which was in closest agreement with the mean of the titres determined on other plates in the same assay was used for the calculation. If there was no such agreement then the assay was repeated for that group of samples. Titres were expressed as the log of the reciprocal of the total dilution at the approximate mid-point of the dose-response curve.

For PT: the concentration of the reference preparation was 250 U/ml. This was diluted by a factor of 20 for use in the ELISA.

Hence, unitage of PT in the test serum was calculated as follows :

$$\frac{\text{Titre of Test Sample}}{\text{Log Mean Titre of IgG Reference Serum}} \times 12.5\text{U} = x\text{U/ml}$$

For FHA: the concentration of the reference population was 400 U/ml; this was diluted 20 times for use in ELISA.

Hence unitage of FHA in the test serum was calculated as follows :

$$\frac{\text{Titre of Test Sample}}{\text{Log Mean Titre of IgG Reference Serum}} \times 20.0U = yU/ml$$

For AGG2,3: unitage in test serum was calculated as follows :

$$\frac{\text{Titre of Test Sample}}{\text{Log Mean Titre of IgG Reference Serum}} \times 400U = zU/ml$$

Seroconversion was defined as a 4-fold or greater increase in the pre-vaccination antibody titre by the age of 9 months.

Calculation of total IgA content:

$$\frac{\text{Titre of test sample}}{\text{Log Mean Titre of IgA Standard}} \times \text{IgA Concentration of Standard}$$

5.6.3 VARIABILITY OF ASSAY

These were determined for IgG antibodies to PT and FHA using sera with different titres, the titres having been correlated using a working standard. The data shown below are for IgG antibodies to FHA.

	GEOMETRIC MEAN	LIMITS (±1 SD)	RATIO OF LIMITS
Intra-plate (3-fold dilution series)			
Serum 1	645	562-740	1.32
Serum 2	14,793	13,862-15,827	1.14
Inter-plate (3-fold dilution series)			
Serum 3	124	99-154	1.55
Serum 4	1,425	1,174-1,731	1.47
Serum 5	7,366	5,924-9,154	1.54

Inter-plate (2-fold dilution series)			
Serum 6	17	15-19	1.32
Sample 7	108	94-123	1.31
Sample 8	269	237-306	1.29

This data indicates that single determinations for each class of antibody to each of the antigens should yield satisfactory results.

5.6.4 CALCULATION OF RESULTS

Geometric mean titres (GMTs) of antibodies against PT, FHA, AGG2,3 and diphtheria and tetanus toxoids were calculated for each group at each point in time when blood samples were collected. GMTs were calculated by taking the antilog of the mean of the log-transformation of non-zero titres. GMTs of antibodies against PT, FHA, and AGG2,3 at months 4, 6 and 9 were compared (Student's t-test on log transformed data). The percentage of subjects demonstrating seroconversion, ie. a ≥4-fold rise in titre from pre-vaccination levels was calculated for each group and compared between groups by chi-square tests.

5.7 DETERMINATION OF NEUTRALIZING ANTIBODIES TO PERTUSSIS TOXIN

5.7.1 INTRODUCTION

The characteristic morphological changes (clustering) in Chinese Hamster Ovary (CHO) cells, observed by Hewlett *et al.* (1983) can be inhibited or neutralized by anti-PT antibodies. This phenomenon is used to estimate the level of PT and of neutralizing antibodies to PT in an *in vitro* neutralization test in microplate cell culture developed and standardized by Gillenius *et al.* (1985).

The assay has several disadvantages -

i. It is slow, morphological change requires approximately 16 hours to develop.

ii. The endpoint (minimum concentration of PT required to produce complete cell

clumping is subjective and tedious to determine.

iii. There is a need for a constant supply of healthy CHO cells in the same phase of growth so as to have the same number of cells in each microwell.

iv. Only neutralizing antibodies to PT are measured by this method, whereas with ELISA, most of the epitopes of PT are detected.

5.7.2 EQUIPMENT AND MATERIAL

1. Neubauer haemocytometer.
2. Carbon-dioxide (CO_2) incubator.
3. Inverted microscope.
4. Vertical laminar flow cabinet.
5. Tissue-culture flasks.
6. Nunclon$^{(R)}$ flat-bottomed τ-radiated disposable plates for cell research work (NUNC 1-67008, Denmark).
7. CHO cell culture. A CHO-K1 cell line maintained by serial passages in GIBCO Minimal Essential Medium (MEM) with 10% (v/v) newborn calf serum was used.
8. *Toxin preparation*: Purified PT was kindly provided by the Research Foundation for Microbiological Diseases (BIKEN), Osaka University, Osaka, Japan. The preparation used contained 200 μg of protein per ml (Table 5.1) and was supplied as a freeze-dried product in 50% glycerol..
9. *Reference serum*: Anti-PT Human IgG. Supplied by BIKEN as a freeze-dried product containing 250 ELISA U/ml PT antibody. Reconstituted to 1 ml in distilled water and diluted 200 times in washing buffer before use in assay.
10. *Culture medium*: GIBCO Minimal Essential Medium (MEM) containing 10% newborn calf serum, 0.11% sodium bicarbonate, 15 mM Hepes Buffer, 0.06% L-glutamine and 25 U/ml of Gentamicin.
11. Medium for Trypsinization of Cells (MTC). Trypsin Versene (TV) - PBS (pH 7.2) containing 0.1% (w/v) trypsin and 0.02% (w/v) EDTA.

12. Giemsa solution (Merck Art 9204).

13. Methanol.

14. Sterile PBS.

15. Trypan blue.

16. Autoclave tape.

5.7.3 METHOD

The methods used were essentially as previously described by Gillenius *et al.* (1985); Granström *et al.* (1985). All manipulations were performed under sterile conditions.

5.7.3.1 Preparation of CHO cells

Confluent CHO cells, obtained in 75 cm^2 tissue culture flasks are collected by rinsing the adherent cells with MTC and leaving 1-2 ml of MTC in contact with the cells for 15 minutes at 37°C.

About 20 ml of culture medium is then added and the non-adherent cells are dispersed gently by pipetting up and down the cell suspension. Five ml of the suspension is placed in a clean tissue culture flask with 35 ml of culture medium and the cells are cultured for 2 days in a CO_2 incubator.

5.7..3.2 Serum clumping inhibition assay (competitive inhibition)

Method:

1. Using a 5-50 µl 12-channel pipette, 25 µl of culture medium is added to every well of a sterile 96-well tissue-culture plate.
2. Using a 5-50 µl single channel pipette, 25 µl of each test serum sample is added to row H of respective columns.
3. With 9 mixes, 25 µl of diluted test sample is then serial diluted one-in-two up the column. The remaining 25 µl is discarded.

4. 25 µl of PT at 18.68 ng/ml (40 MCCs) is added to each well and incubated at 37°C for 3 hours in a humidified incubator. Each plate should include a toxin dilution, toxin positive and negative control wells and a standard positive serum dilution.

5. Once the growth medium from a confluent flask of CHO cells has been aseptically discarded, the cell monolayer is washed with sterile PBS (pH 7.2).

6. Trypsin/versene (2 ml) is then flushed over the cells. Once evenly spread the excess is discarded. With intermittent agitation, the cells are monitored above an inverted microscope. Once most of the cells begin to detach from the plastic, 5 ml culture medium is added and flushed over any remaining cells. The cell suspension is then lightly centrifuged and the supernatant discarded. The pellet is resuspended in 4 ml culture.

7. After a 1/10 dilution in Trypan Blue a viable cell count is carried out in a Neubauer Haemocytometer. On the basis of this the cell suspension is adjusted to 5.4×10^4 viable cells/ml.

8. 200 µl of cell suspension is added to each well of the plate used to carry out sample dilution.

9. An adhesive myalar sheet is then carefully placed over the surface of the plate ensuring that no air may pass into the wells from outside or from adjacent wells.

10. The sealed plate is then incubated in a humidified container at 37°C for 48 hours. Moist paper is placed between plates in order to prevent uneven distribution of cells due to static buildup.

11. The plates are scored as either completely clumped, partially clumped or none clumped. At high dilutions some samples may be seen to be toxic to the CHO cells. Each plate should contain at least 2 standards and at least 1 blank for direct comparison during scoring.

Interpretation of results: The clumping or clustering of CHO cells in plates is scored as follows

T = Toxicity; cells dead hence unable to score.

T/O = No clumping due to endotoxin produced by bacterial or fungal contamination of sera.

O = No clustering, ie. no PT.

1 = Some clustering, ie. 37.5 pg PT.

2 = Intermediate clustering, ie. 75 pg PT.

3 = Clustering of all (100%) cells, ie. 150 pg PT.

Neutralizing titres were expressed as the reciprocal of the highest serum dilution that completely inhibited the PT-induced clustering of CHO cells. A 4-fold or greater increase between 2 samples was considered significant. The anti-PT activity of the reference serum was compared with that of the test sera.

5.7.3.3 PT minimum clumping concentration determination

Method:

1. Sample (vaccine) preparations were diluted in culture medium in serial 10-fold steps using glass tubes. Twenty-five μl of each dilution was further diluted in 2-fold steps directly in the 96-well culture microplates (NUNC 16 7008 Roskilde, Denmark) using a multichannel pipette (Titertek Multichannel, FlowLabs, Irvine, Scotland). The use of glassware was essential at high concentrations of PT due to adherence of the toxin to plastic surfaces.
2. Ten thousand freshly trypsinized CHO cells in 0.2 ml culture medium were then added to each well. After mixing; the plates were incubated at 37°C for 48 hours. All incubations were done in an incubator with an atmosphere of 3.5% (v/v) CO2 in air and a humidity of 98%.
3. The dilution of PT in the test material causing the clustering effect can be observed directly using an inverted microscope or by staining of the cells after 48 hours of incubation with a 2% Giemsa solution for 30 minutes.
4. The end-point titre of toxin titration was set where all cells were clustered. The

sensitivity of the assay was 40-100 pg of toxin per ml, ie. the lowest level of PT which caused all the CHO cells to cluster.

5.8 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND IMMUNO-BLOTTING

5.8.1 INTRODUCTION

This method was used to evaluate the purity of acellular pertussis vaccine antigens, PT and FHA, the specificity of antibody responses to vaccination; and to confirm ELISA data. Antigens were subjected to SDS-PAGE and then electrophoretically transferred from gels to nitrocellulose (NC) paper. Antigen-antibody complexes were then detected using enzyme-labelled anti-immunoglobulin antiserum. PT and FHA do not migrate in a gel buffer system at a basic pH, therefore a multiphasic acid system was used. Details of preparation of reagents for the various steps are given in Appendix 14. All reagents used were electrophoresis grade, unless stated otherwise.

5.8.2 EQUIPMENT

All equipment was purchased from 'Hoefer"

1. Power Supply 500V, 400 MA with 4 outlets. Catalogue No PS 500X.
2. Mini-vertical slab gel unit.
3. Mighty small transfer unit.
4. Deca probe 10 chamber microincubator manifold for antibody screening. Catalogue No PR200.
5. Red rocker
6. Hamilton syringe (5 μl).

5.8.3 PREPARATION OF ANTIGEN FOR ELECTROPHORESIS

The antigen is solubilized or denatured in the presence of SDS which is an anionic detergent.

The SDS unfolds the protein, binds to it, and confers a charge proportional to the length of the polypeptide chain. The charge density is nearly constant for most proteins, hence separation on an SDS-gel is based on gel sieving alone.

Reagents:

1. *Antigens.* Reference PT and FHA freeze-dried antigen preparations supplied by the Research Foundation for Microbial Diseases of Osaka University, Japan and purified from culture supernatant of *B. pertussis.* Details of antigenic properties are shown in Table 5.1.

   PT: 380 μg/ml in 50% glycerol
   FHA: 940 μg/ml in 50% glycerol

   (The vaccine preparation could not be used for electrophoresis because cross-linking in the gel prevents the passage of formaldehyde preparations).

2. 5x Treatment Buffer (TSMG)
   (0125M Tris-Cl pH 6.8; 4% SDS; 20% glycerol, 10% 2-mercaptoethanol)

Method: The antigen is combined with an equal volume of 5x treatment buffer in a test-tube. The tube is placed in a boiling water bath for 5 minutes, then chilled in ice until ready to use. The treated sample may be stored frozen for future runs.

5.8.4 CASTING OF ACRYLAMIDE GELS

The gel consists of 2 layers, an upper narrow "stacking gel" through which the sample passes first, and a lower wider "separating or resolving gel". The stacking gel has large pores and is non-restrictive to the protein sample. The separating gel has smaller pores and allows the protein to be resolved into individual bands according to size.

Reagents:

1. 2% Agar in Running Gel Buffer (1g/50 ml d.$H_2$0).

2. Running Gel Buffers: Separating Gel Buffer (1.5 M Tris-Cl pH 8.8) and Stacking Gel Buffer (0.5 M Tris-Cl pH 6.8)
3. Separating (Resolving Gel) Mixture.
4. Stacking Gel Mixture.
5. Water-saturated n-butanol.
6. Running Gel Overlay (0.375 M Tris-Cl pH 8.8, 0.1% SDS).
7. TEMED (N,N,N',N'-Tetramethlylene diamine).

Procedure:

1. The SE250 unit is assembled using 1.5 mm spacers.
2. The core assembly, a pasteur pipette, a glass plate, and 2% agar in running gel buffer are warmed at 60°C for a few minutes.
3. The core assembly is then placed upright on the glass plate and melted agar is run along the bottom edge and sides of the gel 'sandwiches'.
4. *Preparation of resolving gel*: In a 125 ml side-arm vacuum flask, 60 ml of resolving gel monomer solution is mixed and subjected to deaeration with a vacuum for several minutes. Polymerization initiator and catalyst (TEMED) is added at the last minute. The solution is then pipetted into the 'sandwich' to a level of ± 4 cm from the top.
5. The gel is overlayed with a thin layer of water saturated n-butanol to prevent a meniscus from forming, and to exclude oxygen which would inhibit polymerization on the gel surface. The gel is then allowed to polymerize.
6. The n-butanol is then poured off and the gel surface rinsed with d.$H_2$0 and drained.
7. *Preparation of stacking gel*: The stacking gel monomer solution is prepared and deaerated as before, initiator and catalyst are added and the gel is layered over the resolving gel. A comb is then inserted into the stacking gel, which is then allowed to polymerize (10-15 minutes).
8. The comb is removed and sample wells are rinsed and emptied.

5.8.5 LOADING AND ELECTROPHORESIS OF ANTIGENS BY SDS-PAGE

The antigens are denatured by SDS and separate under electrophoretic conditions as a result of differences in molecular weight and hence size.

Reagents:

Electrophoresis Buffer
Tracking Dye (Bromophenol blue 0.5% w/v in 10% Ethanol).
Antigen samples.

Method:

1. The gel sandwich (core assembly) is placed in the lower buffer chamber of the electrophoresis unit prior to loading the samples.
2. A "well-location decal" is placed against the side of the sandwich, and the sample wells filled, using a 5 μl Hamilton syringe. For 10-well combs and 0.75 mm gels, the sample well volume is 13 μl and 20 μg/well of protein is added.
3. A drop of tracking dye is put into the upper chamber. The two upper buffer chambers are then filled with electrophoresis buffer and the safety lid is placed on the (unit so that the red lead connects with the anode), and the leads are attached to the power supply.
4. The gel is run at 40 mA Constant Current (20 mA/gel) so that the rate of migration will remain constant throughout the run ($\pm$1 hr for 2 x0.75 mm gels).
5. The power supply is turned off when the tracking dye has reached the bottom of the gel (below the surface of the lower buffer chamber). The leads are disconnected and the lid is removed from the unit.
6. The inner core is removed from the lower buffer chamber and poured out the upper buffer chamber by inverting the core.
7. The side clamps are removed and each sandwich is lifted off the core.
8. The gel sandwich is pried open from the bottom with an extra spacer to prevent breaking the ears of the alumina plate. The spacers are then removed and the gel is

peeled off the plate into a tray of transfer buffer or staining solution.

5.8.6 STAINING AND DESTAINING GELS

Stain: Coomassie Blue (CB) R250; (0.05%).

Destaining solution: 10% Glacial acetic acid.

40% Methanol.

Method:

1. Stain gel in 0.05% CB solution overnight by gentle shaking. In the morning change to 0.025% CB solution and leave for about 5 hours.
2. Destain for 10 minutes in destaining solution.
3. Store gel in 40% methanol till required, changing the methanol solution at regular intervals.

5.8.7 ELECTROPHORETIC TRANSFER

Electrophoretic transfer from SDS slab gels to NC paper was performed as described below.

Equipment and material:

Transfer Apparatus: Decaprobe.

Power Supply.

Nitrocellulose Paper (Schleicher & Schüll, Dassel, Germany: 0.45 μm).

Filter paper (Whatman 3M).

Sponge.

Plastic tray.

Transfer Buffer: (72g glycine, 15g TRIS, 1 litre methanol: Made up to a final volume of 5 litre with d.$H_2$0. Stored at 2-8°C. pH 7.8).

Sample diluent: PBST.

Stain: Coomassie Blue (CB) R250 (0.01%).

Method:

1. Fill tray and blotting chamber of apparatus with transfer buffer.
2. Place sponge in tray, lay filter paper over this, and put gel on filter paper.
3. Cover with NC paper and remove air bubbles by rolling a pasteur pipette over it. Cover NC paper with a second sheet of filter paper and sponge.
4. Place 'sandwich' in transfer apparatus, top up with transfer buffer and close chamber.
5. Run gel at 40V (1.2A) for 2 hours at 4°C.
6. Place gel in stain solution to determine if transfer is complete.

5.8.8 DETECTION OF ANTIBODIES TO TEST ANTIGENS BY IMMUNOBLOTTING

Immunoblotting was used to evaluate the specificity of the antibody response to vaccines and to confirm the ELISA data. Antigens subjected to SDS-PAGE were transferred to NC membrane where they reacted with antiserum. Antigen-antibody complexes are detected using an enzyme-substrate colour reaction.

Reagents:

1. Washing buffer.
2. Conjugate - Anti-Human IgG (τ-chain). Peroxidase conjugate SIGMA A6029. Diluted 1:1000In PBST.
3. Substrate buffer - 400 μl of 4-chloronaphthol (10-20 mg in 1 ml methanol).
4. Reference standard antiserum.
5. Blocking buffer.
6. Filter paper.

Method:

1. Following transfer, the NC membrane is placed in the 'DECAPROBE' and blocking buffer is added, followed by rocking for 1 hour. The membrane is then rinsed 3 times in washing buffer.

2. Conjugate control (1 ml) is then added to Well 1 of the DECAPROBE; reference standard (1 ml) is added to Well 2. Serum samples diluted 1:20 (1 ml) are then added to Wells 3-9. The DECAPROBE is incubated overnight at 2-8°C. Immediately prior to use, substrate buffer (colour development solution) is prepared. The NC membrane is immersed in the substrate buffer and incubated for ±15 minutes at 2-8°C. The reaction is stopped by immersing the membrane in distilled water and rinsing. The membrane is dried on filter paper and stored between polyester sheets.

5.9 STATISTICAL METHODS

Statistical analysis of results was carried out by either parametric or non-parametric tests. The latter were utilised when no assumptions could be made on the symmetrical distribution of sample results about the mean and when data was discrete.

The parametric test used was the Student's 't' test and the non-parametric tests employed were either Fishers' Exact Test or the Chi-square.

Calculations on and analysis of antibody titres and ELISA values were performed on logarithmically transformed data. Geometric mean antibody values and standard deviation were calculated. Unpaired and paired 't' tests were used, respectively to compare mean antibody values between clinical groups and change in an individual's values over time. Differences in proportions were evaluated by non-parametric tests. Statistical significance was designated at $p < 0.05$ (2-tailed). Methods used for a particular study are given in it.

TABLE 5.1 Nature of purified pertussis FHA and PT (glycerinated) antigens for use in ELISA.

ANTIGEN	CONTENT TCA-PROTEIN µg/vial	PT-ACTIVITY MOUSE U/ml	PT-ACTIVITY ELISA U/ml	HA-ACTIVITY* HA	HA-ACTIVITY* ELISA U/ml	ET CONTENT** BY LAL ng/ml	PAGE +
FHA	200	<0.5	7.6	6400	7680.0	0.7	1 band
PT	200	256.0	11520.0	NT	7.5	<0.026	1 band

Each vial contained 200 µg protein of 50% glycerinated purified FHA or PT, no preservatives were added.

These products are the same as those sent to the National Bacteriological Laboratory of Sweden for collaborative study.

* Haemagglutinating activity.
** Endotoxin context by limulus amoebocyte assay.
+ Polyacrylamide gel electrophoresis.

TABLE 5.2 Example of computer printout of ELISA data.

29.3.89 Reprint DATA DISC DATA RECALL CODE 1,0 FORMAT PERT1

	1 BLANK	2 REF1	3 TEST1	4 TEST2	5 TEST3	6 TEST4	7 TEST5	8 TEST6	9 TEST7	10 TEST8	11 REF2
A	0.10	0.12	0.10	0.81	0.98	0.11	0.66	1.08	0.13	0.40	0.12
B	0.08	0.15	0.19	1.23	1.47	0.12	1.39	1.91	0.17	0.81	0.14
C	0.08	0.28	0.16	1.87	2.39	0.19	2.23	OVER	0.26	1.63	0.22
D	0.10	0.51	0.30	OVER	OVER	0.38	OVER	OVER	0.51	2.31	0.48
E	0.10	1.10	0.59	OVER	OVER	0.93	OVER	OVER	1.06	OVER	1.17
F	0.13	1.89	1.43	OVER	OVER	1.97	OVER	OVER	1.91	OVER	2.07
G	0.14	OVER	2.37	OVER	OVER	OVER	OVER	OVER	OVER	OVER	2.49
H	0.12	OVER	OVER	2.48	OVER	OVER	OVER	OVER	OVER	OVER	OVER
	TITRE	929	443	39920	58300	778	41470	TOO HIGH RESET	886	18040	1004

DATA DISC, eg. Ph2-A1, Ph2-PT1, Ph2-F1 etc.

Other information in the heading - antigen used, Ig class, Group no

SYMBOL	DATE	DRC	FORMAT	PLOT NO	PLOT TYPE	TITLE	TITRE
+	29:3:89	1.0	PERT1	2	DILUTION	REF1	929
x	29:3:89	1,0	PERT1	3	DILUTION	TEST1	443
◆	29:3:89	1,0	PERT1	4	DILUTION	TEST2	39920
▲	29:3:89	1,0	PERT1	5	DILUTION	TEST3	58305
▼	29:3:89	1,0	PERT1	6	DILUTION	TEST4	778
■	29:3:89	1,0	PERT1	7	DILUTION	TEST5	41470

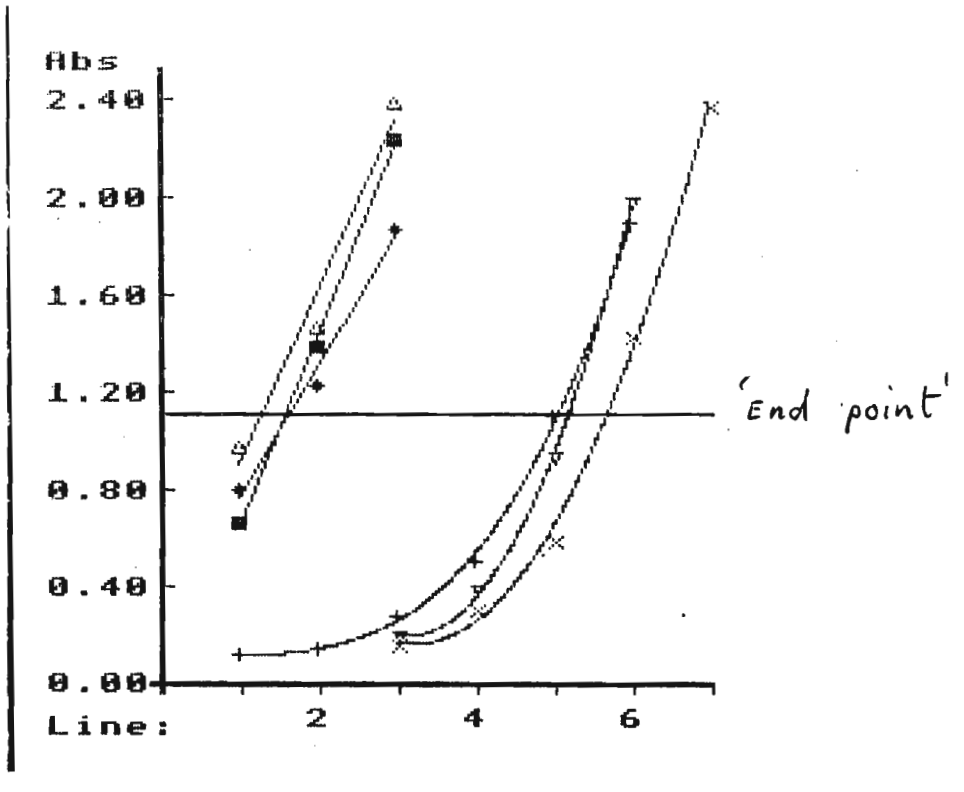


FIGURE 5.1 Plots with software-determined order of fit.

SYMBOL	DATE	DRC	FORMAT	PLOT NO	PLOT TYPE	TITLE	TITRE
+	29:3:89	1.0	PERT1	2	DILUTION	REF1	1085
x	29:3:89	1,0	PERT1	3	DILUTION	TEST1	506
◆	29:3:89	1,0	PERT1	4	DILUTION	TEST2	39920
▲	29:3:89	1,0	PERT1	5	DILUTION	TEST3	58305
▼	29:3:89	1,0	PERT1	6	DILUTION	TEST4	966
■	29:3:89	1,0	PERT1	7	DILUTION	TEST5	41470

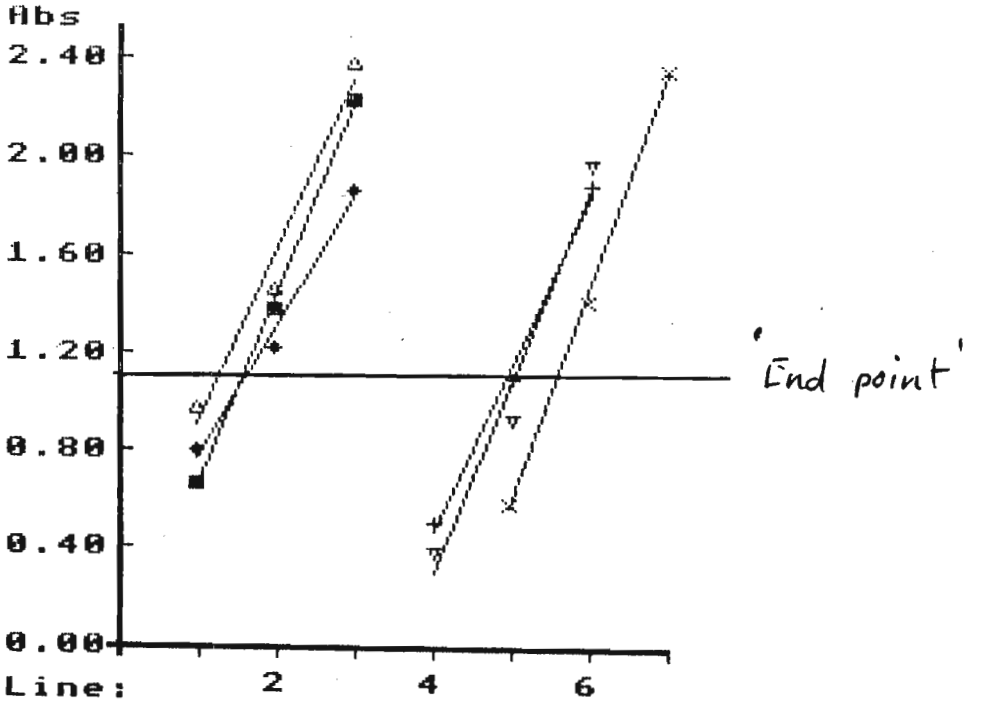


FIGURE 5.2 Plots with linear fit after pruning.

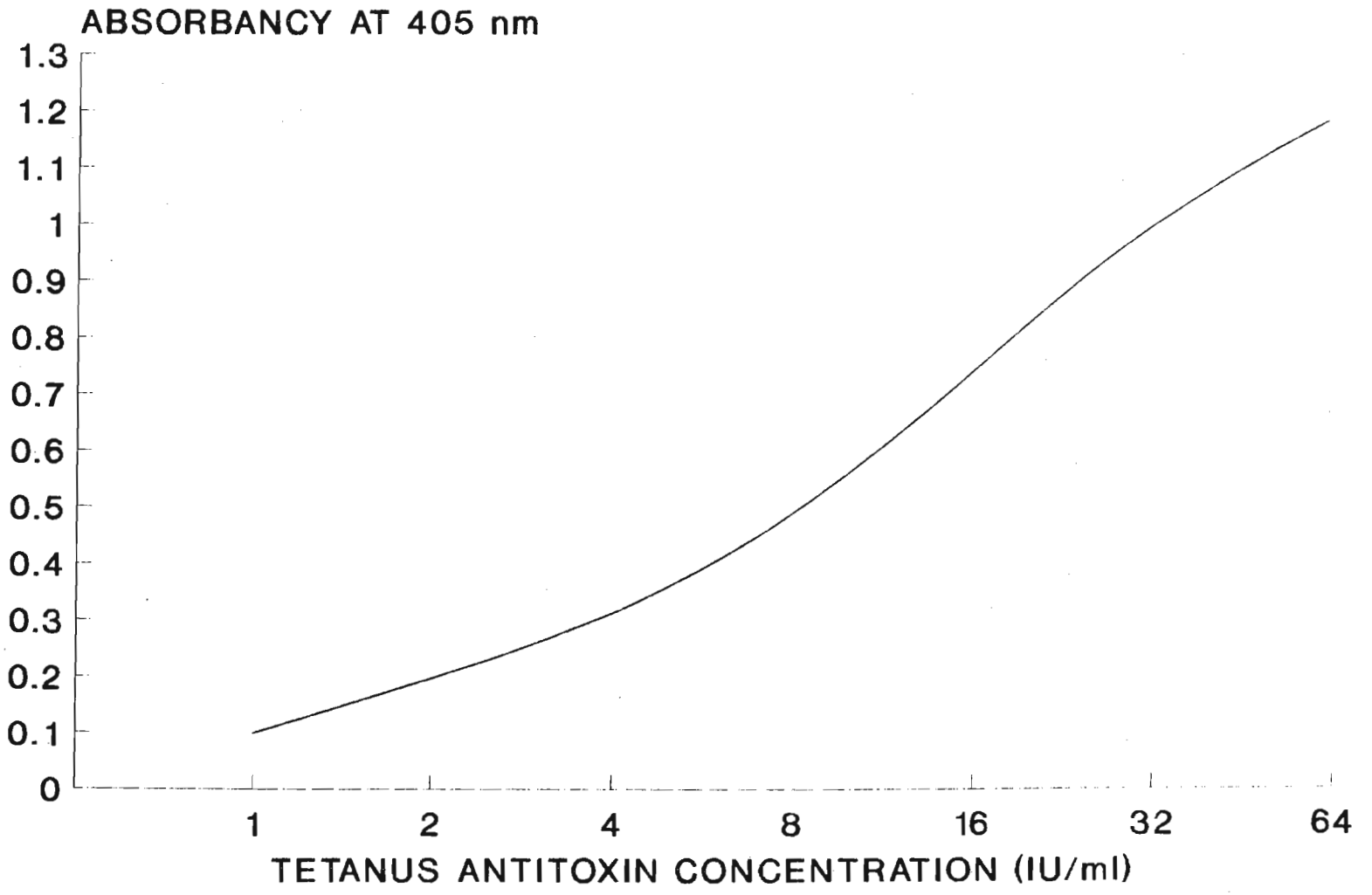


Figure 5.3

Figure 5.3 Standard curve for determination of unitage of tetanus antitoxin in serum samples.

CHAPTER 6

ASPECTS OF WHOOPING COUGH IN SOUTH AFRICA:

A HOSPITAL-BASED STUDY

6.1 OBJECTIVES

To collate available clinical and epidemiological data on whooping cough in South Africa in order to provide background information for undertaking further studies.

6.2 SUMMARY

Whooping cough is not notifiable in South Africa, hence very little data pertaining to the disease is available. Clinical and epidemiological findings from 1525 whooping cough admissions (diagnosed on the basis of clinical criteria) obtained from 6 major infectious disease hospitals around the country for the period 1980-1987/8 are reported. Experience from Durban provided the most detailed information.

Infants and young children were predominantly affected, with 31.3% of cases under 12 months of age and 7.2% less than 2 months. Mortality was disproportionately higher in infancy; 53.2% of deaths were in those younger than 12 months of age. There was a slight female preponderance, both in respect of prevalence and mortality. Patients were admitted with pertussis throughout the year, although there was a peak during the winter months (May to October). The typical whoop accompanied the cough in 55.6% of patients. A raised white cell count was recorded in 66% of patients.

The most commonly detected complication was bronchopneumonia. Nosocomial infections were frequently encountered. The average duration of hospital stay was between 10-13 days. Of those with vaccination records, 26% were unvaccinated, 44% had 1 or 2 doses, and 27% had been fully vaccinated with a whole-cell pertussis vaccine combined with diphtheria and tetanus toxoids (DTP).

The picture, incomplete though it is, reveals a pattern of pertussis similar to that described in other developing countries.

The study reveals huge gaps in our knowledge of this subject in South Africa. More research needs to be done, particularly with respect to improved diagnosis, prevention and treatment; further pertussis should be made a notifiable disease in South Africa.

6.3 INTRODUCTION

Whooping cough (pertussis) is not a notifiable disease in South Africa, except in the City of Cape Town Health Region, hence its incidence, prevalence, severity and transmission remain a matter of conjecture.

Prior to a phase II study of the immunogenicity and safety of a new acellular pertussis vaccine, (see Chapter 8) which appears to be a safer and more effective alternative to conventional whole-cell pertussis vaccines, it was decided to collate available information on whooping cough in South Africa in order to determine baseline data. We intended comparing the pattern of disease seen in this country with that known in other parts of the world.

Data were requested from the admission records of 10 major infectious disease hospitals in the country for the period 1980-1987/88 and were obtained from 6 of these hospitals. It is this information which is presented here. The data from Durban were available in some detail.

6.4 METHODS

6.4.1 SOURCE OF DATA

Admission records for pertussis were requested and obtained from Bethesda Hospital, Ubombo; Livingstone Hospital, Port Elizabeth; City Hospital, Cape Town; Cecilia Makiwane Hospital, Ciskei; Murchison Hospital, Port Shepstone and Clairwood Hospital, Durban. Mortality data were obtained from Dr. L.G.V. Kustner of the Department of National Health and Population Development in Pretoria.

6.4.2 CLINICAL ASSESSMENT

We simply requested information on all cases classified as 'Pertussis' at these centres. Diagnosis at these hospitals was made on the basis of (or recent history of) some of the following: a paroxysmal cough, a whoop, or a cough followed by vomiting with associated subconjunctival haemorrhage or periorbital oedema, marked leucocytosis (> 30,000 mm^3) with relative (60%) lymphocytosis. The precise criteria used are shown under 'Results'. In a recent Swedish study (Blackwelder *et al.*, 1988) 4 categories of case-definition of whooping cough were used -

i. positive bacteriological culture and cough.

ii. positive serology with cough (excluding positive culture).

iii. cough lasting >7 days with spasms, whoops or vomiting in a child having direct contact with a culture-verified case (excluding (i) and (ii).

iv. cough lasting >21 days and whoops (excluding categories (i), (ii) and (iii).

According to the above criteria, all pertussis cases in South Africa would most closely approximate (but not be exactly equivalent to) categories (iii) and (iv). Records are therefore based on suspected cases.

Age and gender, vaccination status, nutritional status, complications, concomitant and associated disease, duration of stay, and date of admission (if available) were recorded for each child admitted to hospital in Durban with whooping cough.

6.5 RESULTS

6.5.1 NUMBER OF CASES

A total of 1525 cases of whooping cough were reported at the 6 hospitals for the period between January 1980 and December 1987 (Table 6.1).

6.5.2 AGE-RELATED PREVALENCE

Age-specific prevalence of whooping cough in 6 infectious disease hospitals in South Africa is shown in Table 6.1. From the information received it was evident that the disease was most prevalent in younger children. Of 1525 cases of whooping cough, 110 (7.2%) occurred in children younger than 2 months of age and 478 (31.3%) occurred in children younger than 12 months of age. Only 7% of cases were over 6 years old. The age-specific prevalence for Durban is shown in Table 6.2.

6.5.3 MORTALITY

Pertussis mortality rates in Durban according to age are presented in Table 6.2 and Figure 6.1. Fifty-one deaths were recorded at the 6 hospitals during the period under review, representing a case-fatality rate of 3.3%. Of these more than half (53%) of the children were below the age of 1 year and 10% less than 2 months of age. Data obtained from the Department of National Health and Population Development (Table 6.3) reported 64 deaths due to pertussis in 1980 and 21 in 1985 for the whole of South Africa. Death due to pertussis was confined to Black and Coloured children.

6.5.4 SYMPTOMS

More than half (55.6%) the patients presented with paroxysmal cough with associated whoop. Absence of the whoop was noted in 44.4% of cases (Table 6.4). There was no significant difference in occurrence of whooping between younger and older children.

6.5.5 WHITE CELL COUNT

White cell counts were available for 425 infants admitted to hospital in Durban. A raised white cell count $\geq 15{,}000$ mm^3 with a lymphocytosis $\geq 50\%$ was recorded in 65.7% of patients, two thirds of whom had counts $> 25{,}000$ cells/μl x^9. One third (33.4%) of patients had a normal count and 0.9% a decreased count (Table 6.5).

6.5.6 SEX DISTRIBUTION

Mortality and morbidity of pertussis was slightly greater in females. Of 1078 cases of pertussis in Durban, Port Elizabeth, Ubombo and Ciskei 627 (58.16%) occurred in females. More than half the 47 deaths (55.8%) occurred in females.

6.5.7 HOSPITAL STAY

Average hospital stay data was available for Cecilia Makiwane Hospital, Ciskei (10.15 days) and Clairwood Hospital, Durban (13.18 days).

6.5.8 COMPLICATIONS AND ASSOCIATED DISEASES

Data on complications were available for 840 patients admitted to hospital in Durban with whooping cough between 1982-1988 (Table 6.6). Bronchopneumonia occurred in 55% of patients, bleeding phenomena in 8.3% and encephalopathy in 4.8%. The age-specific incidence of complications is presented in Table 6.7. Approximately half the children with bronchopneumonia, encephalitis and congestive cardiac failure were younger than 1 year. Haemorrhage was more common in older children.

Many other nosocomial infections (measles, gastroenteritis, tuberculosis) occurred (Table 6.6).

6.5.9 SEASONAL DISTRIBUTION

The mean monthly prevalence of whooping cough admissions in Durban was computed for the 5 year period 1983-1987. Numbers increased during the winter months (May-October) while the lowest prevalence was during January and February (Figure 6.2).

6.5.10 DTP VACCINATION STATUS

Of those with vaccination records, almost half the cases (46.8%) had been unvaccinated, one quarter (25.8%) had partial vaccination and 27.4% had completed primary vaccination (Table 6.8).

6.6 DISCUSSION

Clinical and epidemiological features of whooping cough among hospitalised children in South Africa have been described. Our main findings were that the disease occurred more commonly in younger patients; mortality was disproportionately higher in infancy; slightly more cases and deaths occurred in females; whooping cough was mainly a winter disease; the average duration of stay in hospital was 10-13 days and that vaccinated children remained susceptible to infection. The most frequently employed clinical criteria for diagnosis in South African hospitals were: cough with whoop, bronchopneumonia and a high white cell count in peripheral blood.

The high prevalence and mortality rate of pertussis in children under the age of 1 year in the present study is consistent with findings in other parts of Africa (Morley, 1978; Coovadia & Loening, 1988), and similar to the pattern found in developed countries 50 years ago (Muller *et al.*, 1986).

Other studies have also shown a preponderance of females, and bronchopneumonia as the most common complication (Walker *et al.*, 1981; Krugman *et al.*, 1985). A similar duration of hospital stay (12.6 days) was noted by Conway & Phillips (1989).

There seems little doubt that for several reasons whooping cough remains underdiagnosed in South Africa. The clinical features of the disease vary with age, immune status and the general health of the patient, (eg. infections, nutritional status); as a result pertussis is not easily recognised, which makes diagnosis purely on clinical grounds difficult.

Atypical, and asymptomatic, clinically undetectable pertussis infections have been reported, the occurrence and magnitude of this is unknown. Infants younger than 6 months may present with only apnoea and cyanosis. Most studies have shown that less than 50% of infants with pertussis consistently whoop. Without a history of contact, diagnosis on clinical grounds alone

in these instances is imprecise. The typical whoop accompanied the cough in only 55.6% of patients in the present study.

Laboratory diagnosis of pertussis is also unreliable. Culture of the organism and serologic detection are neither sensitive nor specific. Only 81% of pertussis cases in the United States during 1984-1985 were confirmed by fluorescent antibody techniques, and 40% by culture (Cherry *et al.*, 1988). Routine culture is not undertaken in South Africa and most serologic methods are not easily applicable in the field or even in most hospitals. Diagnosis therefore depends largely on clinical features.

The picture becomes more complicated by the fact that symptoms resembling pertussis can occur as a result of respiratory tract infection by a variety of organisms, such as *Bordetella parapertussis, B. bronchiseptica, Myocoplasma pneumoniae*, adenovirus and influenza virus (Connor, 1970; Nelson, 1975; Granström & Askelöf, 1982). Clearly therefore many cases reported as pertussis in the six hospitals studied are likely to be due to other agents.

A total lymphocyte count in peripheral blood which is raised above normal for the age of the patient, or a markedly increased proportion of lymphocytes, is suggestive of pertussis (Brooksaler & Buchanan, 1976). A raised white cell count was recorded in only 66% of patients in the present study.

Exacerbation of pre-existing illness such as malnutrition, tuberculosis, and congenital heart lesions, makes pertussis a particularly dangerous disease and has led to a mortality of up to 15% of hospitalised cases in Uganda (Bwibo, 1971).

Studies in developing countries have shown case-fatality rates (CFR) of between 1% and 10% in different circumstances (Muller *et al.*, 1986). The CFR for pertussis in the present study was 3.3%, lower than most reports from developing countries suggests, but is still formidable

compared with fatality rates from developed countries. In the majority of recent studies most deaths occurred in patients under the age of 1 year (Nelson, 1978; PHLS Communicable Disease Surveillance Centre, 1983). It is particularly the very young infant who presents with an atypical clinical picture and who commonly has a fatal outcome, but remains undiagnosed. Undiagnosed whooping cough was suggested as one of the ill-defined causes of post-perinatal infant mortality in the United Kingdom (Nicoll & Gardner, 1988).

An enhanced surveillance programme in the United States suggested that passive reporting of whooping cough underestimated clinical cases approximately nine-fold (Halperin *et al.*, 1989).

The low number of pertussis deaths reported to the Department of National Health and Population Development suggests that pertussis may not be a significant cause of infant mortality in South Africa. However, there are considerable deficiencies in notification data in this country. Wyndham (1986) for example, found deaths of black infants under 1 year of age in rural areas in South Africa to be grossly under-reported.

The long duration of whooping cough allows opportunities for other common infections to occur. These combined illnesses or infections make it difficult to determine the precise contribution of pertussis to death rates. This is borne out by the findings in this survey which revealed a frequent association between pertussis and other childhood infections.

In the absence of national notification data, the given unreliability of surveillance data, and the significant percentage of deaths in black infants recorded as due to ill-defined causes, pertussis, in all probability, makes some greater contribution to infant mortality in this country than is generally acknowledged.

There is evidence that vaccination provides greater protection against infection than against disease (Fine & Clarkson, 1987). Transmission of infection is therefore not greatly affected by

vaccination and infection of vaccinated individuals has been reported (Long *et al.*, 1990b). In the absence of specific therapy, vaccination is the only effective means of control available and some measure of protection is imparted by even a single dose of whole-cell vaccine.

The pool of infants and children susceptible to pertussis in a third world middle income country such as South Africa remains large and may be aggravated by poor vaccination coverage and distribution in some regions. Of those with vaccination records in the present study, 26% had received no vaccine, 44% had 1 or 2 doses and only 27% had had the full course. The overall coverage for DTP in South Africa in 1986 was 75% for the full course of 3 injections (Department of National Health & Population Development). Coverage rates varying from 91.4% in the Western Cape (Metcalf *et al.*, 1989) to 57.8% in Khayelitsha, a peri-urban township outside Cape Town (Coetzee *et al.*, 1990) have been reported. Buchmann *et al.* (1987) noted that 56% of infants in Kwa Zulu had 3 doses of DTP, 15% were never vaccinated and that 28-31% of those who received a first dose of DTP did not receive a third dose. Given the variability of regional coverage rates, extrapolation of the above data to the whole country is pointless. Bradshaw *et al.* (1987) have made the point that data for blacks have serious quantitative and classification deficiencies not least because vaccine coverages are based on vaccine distribution.

In 1988, only 36% of children in Africa (excluding South Africa) were vaccinated with the third dose of DTP (WHO, 1988).

Considerable controversy surrounds the nature and magnitude of protection afforded by whole-cell pertussis vaccines. Numerous studies on conventional whole-cell pertussis vaccine have reported a wide range of efficacy rates ranging from nil in Sweden in the 1970's to approximately 95% (Fine & Clarkson, 1987).

Increased incidence of pertussis following reduced vaccination rates in several countries in the

1970's illustrate that the vaccines provided some degree of protection in those specific situations (Kanai, 1980; Centers for Disease Control, 1982). However, use of the vaccine has not been reintroduced in Sweden and Japan because of concern over adverse reactions (Romanus, 1979).

The efficacy of the pertussis component of the South African DTP has not been established and no studies on $\widehat{VE}$ have been published. Whooping cough notifications in Cape Town dropped precipitously in the 1950's after the introduction of pertussis vaccination (Cape Town City Council, 1989), providing evidence of the effectiveness of the South African pertussis vaccine. However recent localised outbreaks of the disease in urban areas which have been reported, eg. Cape Town (Strebel *et al.*, 1989) could be due either to poor vaccination coverage or low vaccine efficacy.

Antibody responses to PT, FHA, AGG2,3 and 69kDa outer membrane protein following whole-cell vaccine have been demonstrated in other series. The lack of antibody production against the major protective antigens of *B. pertussis*, PT and FHA, in recipients of South African DTP (Ramkissoon *et al.*, 1989) may be indicative of poor vaccine efficacy. However, this study was not designed to determine the protective effect of the vaccines used and the acquisition of IgG antibodies to PT and FHA have not been shown to correlate to clinical immunity or vaccine efficacy.

Epidemiological and laboratory studies are currently being undertaken by the South African Institute of Medical Research to establish evidence of $\widehat{VE}$ (Schrire & Koornhof, 1989).

A prospective study of $\widehat{VE}$ in the Cape (Metcalf *et al.*, 1989) determined from the secondary attack rates in household contacts, gave a point estimate of 84%, with a 95% confidence interval of 9-100%. Given this wide confidence interval, this figure of $\widehat{VE}$ lacks statistical power and can therefore be interpreted in different ways by different people.

The hotly debated issue in some developed countries as to whether the benefits of routine pertussis vaccination in infants outweigh the risks because of the reported serious adverse reactions to vaccine is of slightly less relevance in South Africa since the incidence of illness, hospitalisation and death from pertussis infection appears to outweigh the presumed risk of vaccine-associated encephalopathy.

From the few available records of whooping cough $\hat{VE}$, attack rates and mortality in African countries (Morley *et al.*, 1966; Muller *et al.*, 1984) the pertussis risk in early infancy emphasises the need for vaccination as early in life as possible to reduce incidence as well as severity of the disease.

Serious consideration needs to be given to establishing whooping cough as a notifiable disease in South Africa, notwithstanding the limitations of diagnostic accuracy. The diagnosis could be based on criteria selected by a local panel of experts from those which have been accepted universally. Regular and countrywide notifications will *inter alia* contribute to an understanding of the epidemiology of pertussis-like syndromes, will give some idea of the efficacy of current vaccination programmes, and will provide baseline data on which can be based the introduction of improved vaccines. Furthermore, such notification will strengthen the specificity of mortality rates for infancy and childhood. There is clearly a need for further research into aspects of this neglected disease, particularly with regard to improved diagnostic procedures applicable in the primary and secondary care settings, prevention of the disease and more effective treatment.

TABLE 6.1 Whooping cough admissions, deaths and age-specific prevalence in six infectious disease hospitals in South Africa.

SOURCE	Number of cases	Number of deaths	Case fatality rate (/1000)	Number of cases <2 mths (%)	Number of cases <1 year (%)	Average age in months (range)
Durban 1982-1987	840	40	47.62	60(7.1)	345(41.1)	26.31(<1-96)
Port Shepstone 1980-1987	53	0	0	0(0)	20(37.7)	32.71(3-108)
Ciskei 1982-1987	86	3	34.88	26(30.2)	52(60.8)	22.14(1-96)
Ubombo 1982-1987	73	4	54.79	2(2.7)	8(10.9)	--- (2-72)
Port Elizabeth 1981-1987	71	1	14.08	22(30.9)	53(74.6)	12.52(<1-84)
Cape Town 1986-1987	394	3	7.64	---	---	---
TOTAL	1525	51	33.44	110(7.2)	478(31.3)	<1-108

TABLE 6.2 Age-specific prevalence, morbidity and mortality; of whooping-cough at Clairwood Hospital, Durban (1982-1988).

Age in months	Percentage of cases (n)	Percentage of deaths (n)	Contributory cause of death (n)
0≤3	10.8(63)	0	---
>3≤6	9.1(53)	12(3)	PEM 1 BPN 1 ENC 1
>6≤12	19.5(114)	48(12)	MEA 5 ENC 4 BPN 2 CCF 1
>12≤36	35.0(205)	24(6)	ENC 4 BPN 2
>36≤72	18.6(109)	12(3)	BPN 2 ENC 1
≥72	7.0(41)	4(1)	MEA 1
TOTAL	100(585)	100(25)	ENC 10 BPN 7 MEA 6 PEM 1 CCF 1

PEM = protein energy malnutrition.
BPN = bronchopneumonia.
CCF = congestive cardiac failure.
MEA = measles.
ENC = encephalitis.

TABLE 6.3 Notification of whooping cough deaths during 1980 and 1985 in South Africa.

AGE IN YEARS	<1	1	2	3	4	>4	TOTAL
1980							
Black	21	9	1	1	2	6	40
White	-	-	-	-	-	-	0
Asian	-	-	-	-	-	-	0
Coloured	3	10	1	2	2	6	24
TOTAL	24	19	2	3	4	12	64
1985							
Black	5	5	3	1	1	2	17
White	-	-	-	-	-	-	0
Asian	-	-	-	-	-	-	0
Coloured	2	0	0	0	0	2	4
TOTAL	7	5	3	1	1	4	21

(Personal communication. Dr. L.G.V. Küstner, Department of National Health and Population Development).

TABLE 6.4 Clinical symptoms of whooping cough in hospitalised children.

AGE in months	% OF CHILDREN WITH COUGH AND WHOOP n=326	% OF CHILDREN WITH COUGH ONLY n=359
0≤3	5.6	4.8
>3≤6	3.8	5.1
>6≤12	10.9	8.6
>12≤36	20.5	14.4
>36≤72	10.9	7.7
≥72	3.9	3.8
TOTAL	55.6	44.4

TABLE 6.5 White-cell counts in hospitalised children with whooping cough, Durban (1980-1988).

AGE in months	RAISED COUNT (%)	DECREASED COUNT (%)	NORMAL COUNT (%)
$0 \leq 3$	5.9	0	5.9
$> 3 \leq 6$	3.8	0	3.8
$> 6 \leq 12$	14.2	0.2	5.9
$> 12 \leq 36$	23.7	0.7	8.9
$> 36 \leq 72$	12.5	0	6.6
≥ 72	5.6	0	2.3
TOTAL	65.7	0.9	33.4

The normal range of total white cells ($x10^9/l$) was taken as being 5.0-18.5 for 0-3 months; 5.0-17.5 for >6 to 6 months; 11.0-17.5 for >6 months to 3 years and 5.0-14.5 for >3 years.

TABLE 6.6 Complications, associated diseases and signs in 840 children admitted to hospital with whooping cough (1982-1987).

	PERCENTAGE OF CHILDREN	(n)
Complications		
Bronchopneumonia	55.0	462
Haemorrhage	8.3	69
Encephalopathy	4.8	40
Congestive cardiac failure	0.8	7
TOTAL	68.9	57.8
Associated diseases		
Malnutrition	6.2	52
Measles	6.1	51
Oral herpes	2.9	24
Gastroenteritis	1.8	15
Chicken pox	1.3	11
Anaemia	0.7	6
Mumps	0.6	5
Tuberculosis	0.5	4
Measles and bronchopneumonia	0.4	3
Hepatitis	0.2	2
Bronchopneumonia and kwashiorkor	0.2	2
Ventricular septum defect	0.2	2
Syphilis	0.1	1
Cardiomyopathy	0.1	1
Measles and tuberculosis	0.1	1
Bronchopneumonia and tuberculosis	0.1	1
Amoebic liver abscess	0.1	1
TOTAL	21.6	182

TABLE 6.7 Age-specific prevalence of whooping cough complications in 585 children at Clairwood Hospital, Durban (1982-1988).

AGE in months	PERCENTAGE OF CHILDREN WITH COMPLICATIONS (n)				
	Bpn*	Encephalitis	CCF**	Haemorrhage	
				Epistaxix	Sub-conjunctival
0≤3	7.9(46)	0.3(2)	0	0	0
>3≤6	6.5(38)	0.3(2)	0	0	0
>6≤12	16.2(95)	1.7(10)	0.5(3)	0.3(2)	0.3(2)
0≤12	30.6(179)	2.3(14)	0.5(3)	0.3(2)	0.3(2)
>12-36	22.6(132)	3.9(23)	0.3(2)	0	3.2(19)
>36-72	16.6(97)	0.3(2)	0.2(1)	0.9(5)	3.9(23)
≥72	4.1(24)	0	0.2(1)	0.7(4)	2.4(14)
TOTAL	73.8(432)	6.7(39)	1.2(7)	1.9(11)	9.8(58)

* Bronchopneumonia.
** Congestive cardiac failure.

TABLE 6.8 Vaccination status of 472 hospitalised children with pertussis infection (Durban, 1982-1987).

AGE in months	NUMBER OF DOSES nil	1or2	3
0≤3	91.7*	8.3	0
>3≤6	54.5	36.4	9.1
>6≤12	48.5	31.3	20.2
>12≤36	39.5	25.9	34.6
>36≤72	29.3	29.3	41.4
≥72	25.0	18.8	56.2
TOTAL	46.8	25.8	27.4

* = %

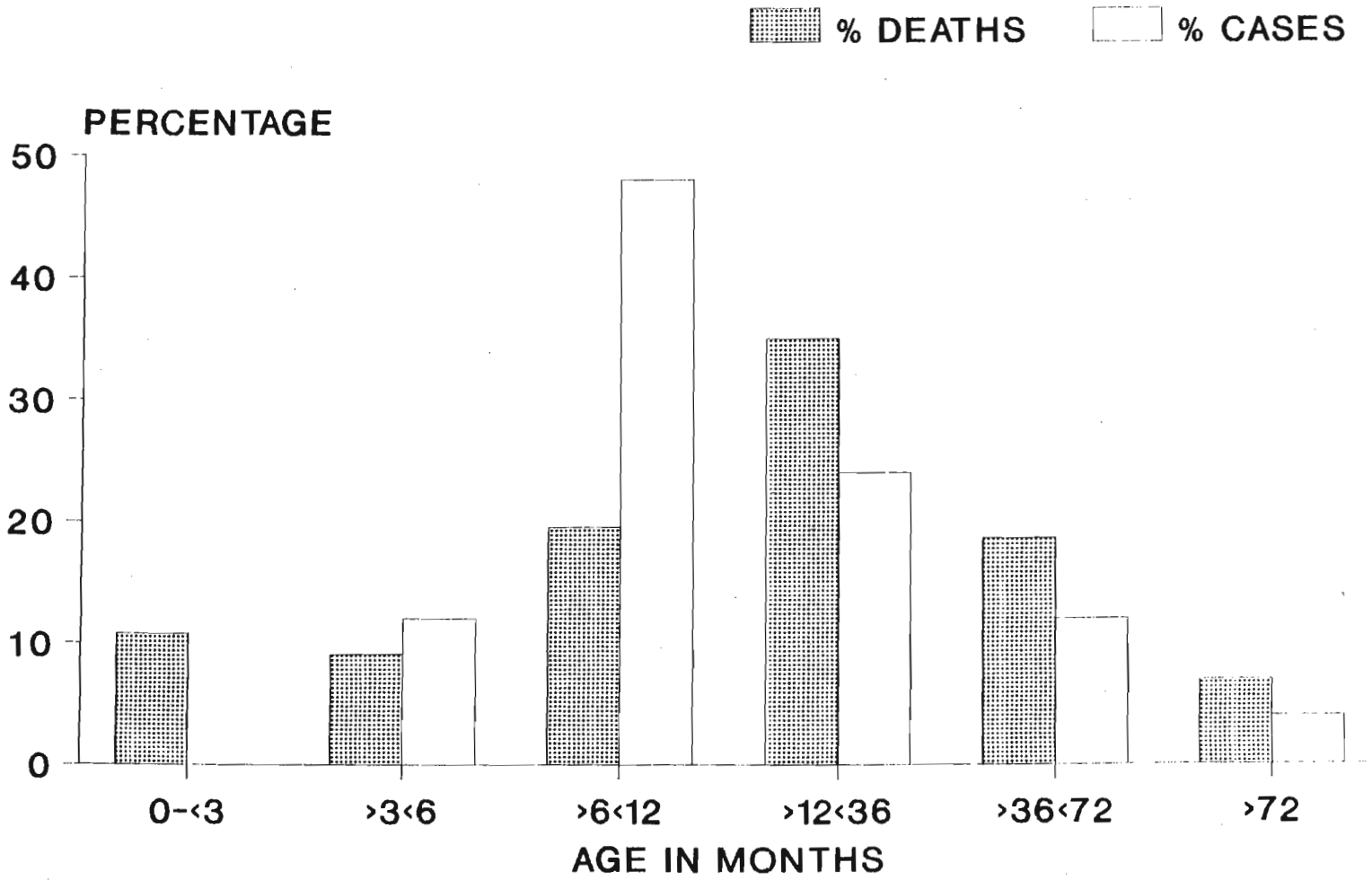


FIGURE 6.1 Age related incidence of whooping cough mortality in South Africa.

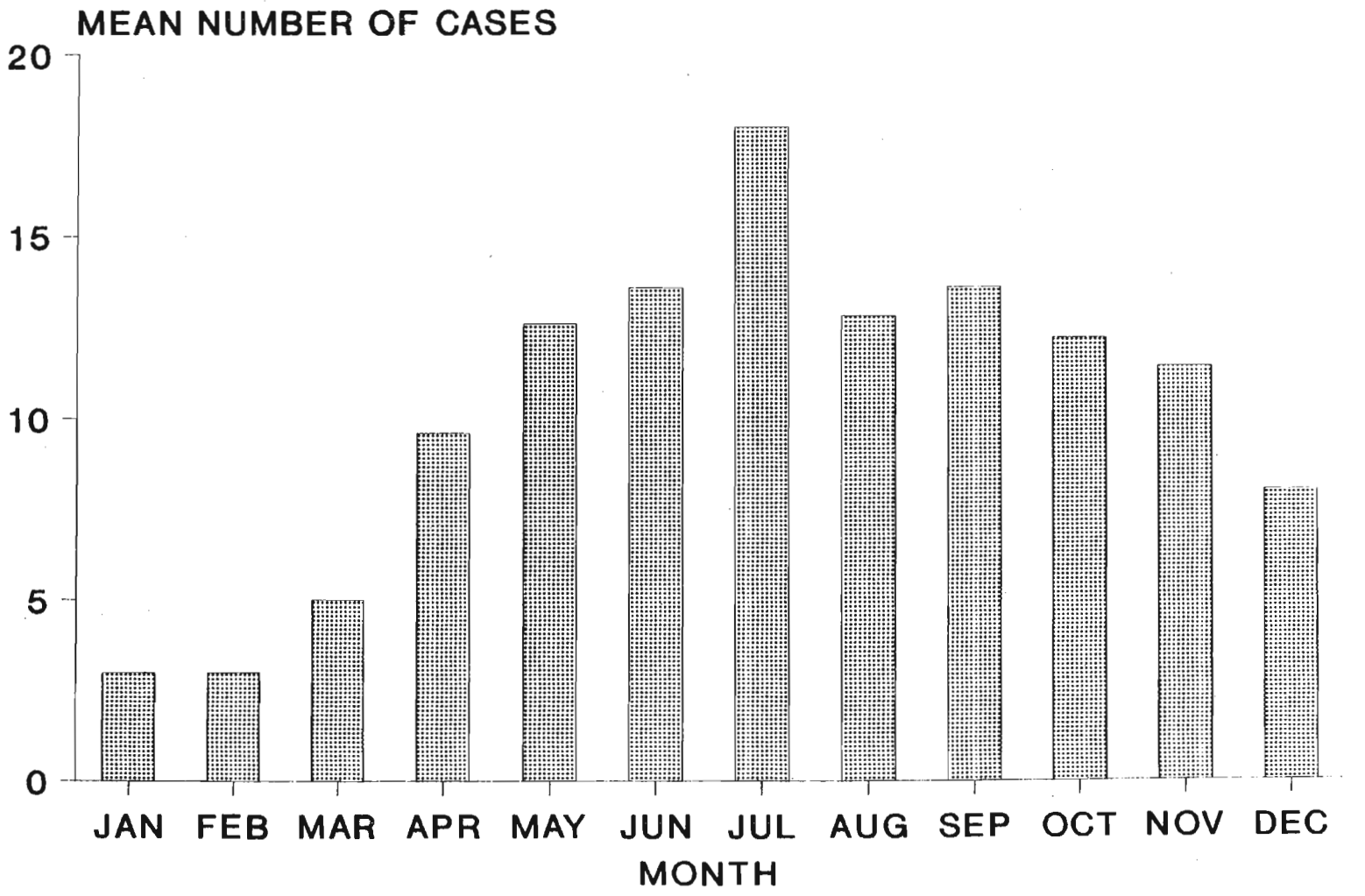


FIGURE 6.2 Mean monthly distribution of hospitalised pertussis cases in children in Durban (1982-1987).

CHAPTER 7

WHOLE-CELL PERTUSSIS VACCINE IN BLACK SOUTH AFRICAN INFANTS

7.1 OBJECTIVES

The objectives of this study of the South African combined trivalent diphtheria, tetanus, whole-cell pertussis (W-DTP) vaccine were -

1. To evaluate the immunogenicity of the vaccine by measuring specific antibody levels against *B. pertussis* antigens in serum samples collected before, during and after the primary vaccination course.

2. To determine the short-term safety and reactogenicity of the vaccine from the beginning of the primary vaccine course (month 2) until the age of 9 months.

7.2 SUMMARY

Whole-cell pertussis vaccine currently used in South Africa has not been adequately evaluated for post-vaccination events and immunogenicity. A trial of this vaccine combined with diphtheria (D) and tetanus (T) toxoids as was undertaken in 115 African babies who received primary vaccination at 2, 4 and 6 months of age.

Serological IgG responses to 3 major antigens of *B. pertussis*, filamentous haemagglutinin (FHA), pertussis toxin (PT) and fimbrial agglutinogens 2 and 3 (AGG2,3)) were evaluated by ELISA in sera obtained at birth, and prior to vaccination at 2, 4 and 6 months, and at 9 months.

Surprisingly, after 3 doses of W-DTP responses to PT and FHA were found merely to restore PT and FHA IgG antibody titres to those found in cord blood, which questions the immunogenicity of the South African product. In contrast with the positive increases in these antibodies found in other series of whole-cell vaccination, the anti-PT seroconversion rate was only 19.0% and the anti-FHA rate only 24.1%. High levels of anti-AGG2,3 were produced with 67.2%

seroconversion.

The frequency and nature of post-vaccination events were recorded for 14 days after each dose. Fever 3.2%) and excessive crying (2.4%) were the most frequently occurring minor events. The rate of neurological post-vaccination events (without sequelae) during the brief follow-up period was considerably high; 2 hypotonic-hyporesponsive episodes (8.03/1000 doses) and 1 convulsion (4.02/1000 doses). The small study size however prevents a definite comment on the incidence of these events following whole-cell pertussis vaccination.

Significant pertussis antibody levels were found in maternal and cord sera, with levels in the latter frequently being higher, suggesting a high prevalence of infection amongst child-bearing women as well as active transplacental transfer of antibody. Three cases of pertussis infection occurred during the study period. Only one of the subjects had completed primary vaccination. In view of these findings, the need for proper efficacy and safety studies of the currently used W-DTP vaccine are urgently indicated in South Africa.

7.3 INTRODUCTION

Pertussis (whooping cough) remains a major cause of morbidity and mortality in the very young more especially in the Third World. According to World Health Organisation estimates, 60,000 deaths due to pertussis occur yearly in Africa, virtually all in unvaccinated infants (UNICEF, 1988). Some hospital-derived evidence (Chapter 6) suggests that the incidence of morbidity and mortality of the disease in South Africa as elsewhere are highest in infancy. Effective and safe preventive measures against pertussis are therefore required .

Conventional whole-cell pertussis vaccines presently used in most parts of the world consist of inactivated cells of *B. pertussis* treated with heat or formalin to render them non-infective and are therefore a mixture of antigens, some of which, while essential for protection, also produce

adverse reactions.

The safety and efficacy of whole-cell pertussis vaccines have been the subject of discussion in medical publications for the past decade. These are considered the most reactive of childhood vaccines used in routine vaccination schedules, although the possible contribution of the diphtheria and tetanus components of triple vaccine is frequently ignored. Whole-cell vaccines are associated with a wide range and high rate of adverse effects. Local and systemic reactions (crying, fever, swelling, induration) are common, and although less common, the occurrence of temporally associated neurological disorders and sudden death have been reported (Cody *et al.*, 1981). The exact incidence and relationship of these reactions to pertussis vaccine is not established. Use of the vaccine was discontinued in the 1970's in Japan after the sudden death of recently vaccinated infants, and in Sweden where protection after vaccination with unadsorbed vaccine was poor and side-effects unacceptable.

Efficacy rates ranging from nil to 95% have been reported throughout the world (Fine & Clarkson, 1987). The best evidence that whole-cell vaccines are indeed effective in reducing morbidity and mortality in pertussis is the increased incidence of the disease and epidemics that follow withdrawal of vaccine usage (Centres for Disease Control, 1982a,1982b; Kanai, 1980). Despite concerns it is widely recognised that the benefits of effective vaccination outweigh the risks (Koplan *et al.*, 1979; Miller *et al.*, 1982).

Pertussis vaccines vary from country to country, as do vaccination schedules and recommendations, and clinical monitoring techniques. Vaccine-efficacy appears to be good in some areas of South Africa but for other areas there is no information and recent outbreaks of the disease have occurred in Cape Town and Durban (Metcalf *et al.*, 1989). The disease is not notifiable in South Africa and consequently no hard data on efficacy have been reported. There has also been no assessment of responses to the South African whole-cell pertussis vaccine. This study was therefore undertaken to assess post-vaccination events and to measure serum

IgG antibody responses to 3 major antigens - PT, FHA and AGG2,3 - in recipients of this vaccine administered as a 3-dose primary series at 2, 4 and 6 months of age. Maternal antibodies were also measured since little is known about maternofetal transfer of pertussis antibodies, especially in African countries where inhibition of placental transfer might occur for a variety of reasons.

7.4 SUBJECTS AND METHODS

Details of patients and methods are provided in Chapters 4 and 5, hence only a very brief account is given here in this section so that the chapter can be read as a whole.

7.4.1 STUDY DESIGN

This is shown in Figure 7.1. This was an open unblinded study. Infants were recruited into the Phase II providing that written informed parental consent was obtained and that they resided in an area suitable for follow-up. The subject information sheet in shown in Appendix 2. Antenatal history and details of family circumstances were ascertained at this time. Babies were assigned to 1 of 3 vaccination groups sequentially in their order of admission to the study. Each group comprised 115 children. Two groups received acellular pertussis vaccine (A-DTP) and 1 group received whole-cell pertussis vaccine (W-DTP) (Chapter 8) as detailed below (Section 7.4.6). Mothers and nursing staff were unaware of which vaccine was given. The study was reviewed and approved by the Ethics Committee of the University of Natal. No unvaccinated controls were studied for ethical reasons.

7.4.2 STUDY POPULATION

In the 3 month period from March to May 1988, 115 healthy full-term newborn infants from Kwa Mashu, a peri-urban suburb of Durban inhabited exclusively by blacks, were enrolled in this study. The subjects formed part of a larger open unblinded phase II trial of the antibody responses and symptoms to the acellular Japanese J-NIH-6 pertussis vaccine (Chapter 8)

(Ramkissoon *et al.*, 1988). The subjects received BCG and trivalent oral polio vaccine (TOPV) at birth and were asked to return at 2, 4, 6 and 9 months of age. Whole-cell DTP and TOPV were administered at the time of the 2, 4 and 6 month visits in accordance with current WHO recommendations. Measles vaccine was administered at 9 months of age. Blood sampling was prior to vaccination at each visit.

Three deaths which were not vaccine-related occurred before the subjects were 2 months old. All had been assigned to the whole-cell vaccine group and had therefore received only BCG and TOPV at birth. Of the 112 remaining subjects, 91 (81%) returned at 2 months of age, 83 (74%) at 4 months, 75 (67%) at 6 months and 60 (54%) at 9 months of age.

Exclusions for each injection:

1. Infants with known or suspected history of pertussis, diphtheria or tetanus.
2. Infants with known or suspected impairment of their immune function or with major congenital malformations or serious chronic disorders.
3. Infants with a confirmed or suspected underlying neurological disorder or history of neonatal seizures.
4. Infants with a pronounced reaction (eg. seizures, collapse or shock-like reaction, abnormally high temperature) after a previous dose.

7.4.3 CLINICAL FOLLOW-UP

Each child was weighed, measured, and underwent physical examination by a paediatrician before being vaccinated at every visit. Children with acute illnesses were vaccinated after the illness subsided. The children were also carefully monitored for intercurrent illnesses by a community nurse up to 9 months of age. All concomitant medications, anthropometric data, clinical signs and vaccination data were recorded at each clinic visit.

7.4.4 NUTRITIONAL STATUS

Nutritional status of subjects was assessed at each clinic visit by the anthropometric indices of length- and weight-for age, clinical features of protein-energy malnutrition, and vitamin or trace element deficiencies. The US National Center for Health Statistics Research Population was used as a standard. Children with a weight-for-age less then the third percentile were considered underweight and children with a length-for-age less than 90% of the median were considered stunted (Waterlow *et al.*, 1977).

7.4.5 EVALUATION OF POST-VACCINATION EVENTS

An illustrated record sheet was given to all parents to record post-vaccination events for a period of at least 14 days after each vaccination (Appendix 1). Detailed instructions pertaining to interpretation of the illustrations were conveyed in the native language (Zulu) by a registered nurse. Eight symptoms and signs were registered, viz. fever, loss of appetite, excessive crying, fretfulness, convulsions, hypotonic-hyporesponsive episodes, swelling or induration at injection site (irrespective of size), or other symptoms regarded as possible vaccine-associated. The parents were instructed to inform the paediatrician-in-charge of the trial immediately in case of serious reactions (usually through the community nurse). Investigator information and addresses were provided in Zulu and English (Appendix 3). Parents were not asked to take temperatures. Fever was measured qualitatively (parents' impression) because of the limited educational background of the population. Convulsions and hypotonic-hyporesponsive episodes were explained in some detail by the nurse. Before the second and third doses of DTP symptoms that had occurred following the previous injection were assessed and evaluated.

7.4.6 BLOOD SAMPLING

A sample of mother's blood and cord blood were obtained for each infant at birth. In addition, whole blood samples were also taken at 2, 4 and 6 months of age, immediately before vaccination. A fifth sample was taken at 9 months of age. Sera obtained from these samples were coded and frozen at -20°C until antibody assays could be performed. All sera from one

individual were tested in the same assays on the same day. In some cases the sample was not sufficient to carry out all the tests required and therefore the numbers of samples giving rise to the data shown in the tables are not uniform.

7.4.7 VACCINE AND VACCINE ADMINISTRATION

The adsorbed whole-cell DTP vaccine used was manufactured by the South African Institute for Medical Research, Johannesburg. The vaccine was made up of 50 Lf/ml Diphtheria toxoid, 12 Lf/ml tetanus toxoid, 20,000 million *B. pertussis*/ml, 2.5 mg/ml aluminium phosphate (adjuvant) and 0.01% thiomerosol (preservative). The vaccine was administered in 0.5 ml doses by intramuscular injection in the left thigh at 2, 4 and 6 months of age. A single lot (No A595) was used. The date of manufacture was 1 January 1988 and date of expiry 1 January 1990. Appendix 9 shows the package insert enclosed by the manufacturers' detailing vaccine specifications.

7.4.8 SEROLOGIC ASSAYS

IgG antibodies to FHA, PT and AGG2,3 were assayed by ELISA at the Centre for Applied Microbiology and Research, Public Health Laboratory Services, Porton, United Kingdom by the author. The ELISA procedure used was essentially as described by Rutter *et al.* (1988). Concentrations of antigens and conjugate used were determined by chequerboard titrations (Chapter 5)

Antigens: FHA and PT used as antigens in the ELISA were purchased from the Research Foundation for Microbial Diseases of Osaka University, Japan. Co-purified AGG2,3 was provided by Dr. A. Robinson, Porton, United Kingdom.

Reference serum: The Japanese Reference Pertussis Anti-Serum (human) was a gift from the Research Foundation for Microbial Diseases of Osaka University, Japan. It was supplied from a single lot and contained 250 ELISA Units of Pt-IgG antibody and 400 ELISA Units of FHA IgG

antibody to pertussis per ml. The reference serum was assigned a value of 400 ELISA units/ml of anti-AGG2,3.

Calculation of results: Results were expressed as ELISA U/ml. The unitage of the test serum relative to the reference serum was calculated by means of parallel-line assays.

7.4.9 STATISTICAL ANALYSIS

Serology: All serologic results were log transformed to geometric values before statistical analysis. Geometric mean titre (GMTs) of antibodies against PT, FHA and AGG2,3 were calculated for each group at each point in time when blood samples were collected.

GMTs of antibodies against PT, FHA and AGG2,3 were compared after each dose by Student's t-test. GMTs of infants infected with pertussis were excluded from the analysis of serologic responses. Seroconversion, ie. the percentage of infants demonstrating a serologic response was calculated, ie. a $\geq$4-fold increase over pre-vaccination (2 month) titres. Percentage seroconversion was compared between groups at 6 and 9 months of age by chi-square tests.

Post-vaccination events: The incidence of individual solicited symptoms over each post-vaccination follow-up period (14 days) per group was calculated, ie. for each dose.

The incidence of selected symptoms was compared between groups (chi-square). The incidence of infants with and without symptoms was determined per 1000 doses of vaccine administered.

7.5 RESULTS

7.5.1 CLINICAL RECORD

Three cases of subclinical pertussis occurred in female infants. The first case occurred in a

subject 2 months after the first dose of vaccine was administered, ie. at 4 months of age. The second case occurred in an unvaccinated infant who was 2 months of age. The third case occurred in an 8-month old infant who had been fully immunised (3 doses of vaccine). No clinical signs of pertussis occurred in these infants. Diagnosis was made retrospectively and based on the rapid ($\geq$ 4-fold) and marked rise in levels of all 3 pertussis IgG antibodies between 2 consecutive serum samples, followed by a rapid decline in levels at the time of postulated infection. To eliminate the possibility of inadvertent vaccine administration ELISA for D&T antibodies were performed. Further details of subclinical pertussis are given in Chapter 9.

Incidence of intercurrent illnesses in recipients of W-DTP from birth to 9 months of age are shown in Table 7.1. The highest incidence of common childhood illnesses occurred between 2 and 4 months of age (29.07% of infants with illness. Infections of the upper respiratory tract were the most commonly occurring (37.2% of illnesses), followed by skin infections (30.2%) and diarrhoea (8.1%).

7.5.2 NUTRITIONAL STATUS

A total of 6 children were found to be malnourished at various times during the study. Two children were underweight at birth but recovered before the first vaccination at 2 months of age. One child was underweight and stunted at 2 months of age. Two children were underweight at 6 and 9 months of age. Their nutritional status had all reverted to normal by the next clinic visit. One child developed protein-energy malnutrition at 6 months of age and was still malnourished at 9 months of age. Further details of the effect of vaccination in malnourished infants are given in Chapter 11.

7.5.3 POST-VACCINATION EVENTS

Parental evaluation of post-vaccination events occurring in the 14 days after vaccination was available for 249 doses of whole-cell pertussis vaccine. The number of post-vaccination events was very low (Table 7.2). A total of 11 infants experienced an event during the study. These are

noted in Table 7.3. These subjects appeared normal on subsequent clinical examination. Fever (32%) and excessive crying (2.4%) were the most frequently occurring systemic reactions. Fever, excessive crying, irritability and hypotonic-hyporesponsive episodes occurred more frequently after the first dose than after the second or third doses. No infant had a local symptom (swelling or induration) at the injection site, which is most unusual. Other than local symptoms post-vaccination events were transient and all responded to simple treatment at home without any sequelae.

Three neurologically-associated post-vaccination events occurred. One male infant experienced febrile convulsions (4.03/1000 doses) as well as a cough with wheeze, within 7 days after the third dose of vaccine. He was admitted to hospital 30 days later with measles and bronchopneumonia. No neurologic sequelae resulted. Two hypotonic-hyporesponsive episodes (collapse, shock) ie. 8.03 cases/1000 doses occurred within 7 days after the first dose of vaccine. It was not possible to ascertain the exact time after vaccination at which these 3 events occurred.

7.5.4 TRANSPLACENTAL TRANSFER OF PERTUSSIS ANTIBODIES

Antibodies to all 3 antigens tested were present in relatively high quantities in maternal sera and often detected in higher quantities in cord blood; (hence active transfer across the placenta occurred) (Table 7.4). All mothers had relatively high levels of IgG antibody to FHA and AGG2,3. Only 7% of mothers had very low levels of IgG anti-PT, ie. <5U. Further details of transplacental transfer are given in Chapter 10.

7.5.5 SEROLOGICAL RESPONSES TO VACCINATION

Filamentous haemagglutinin (FHA) and pertussis toxin (PT): Geometric mean titres (GMTs) of IgG antibodies to FHA and PT in cord blood and at 2, 4, 6 and 9 months of age are shown in Table 7.2 and Figures 7.2 and 7.3).

The anti-FHA GMT was relatively high at birth and then declined steadily throughout the period of vaccination. After 3 doses of the vaccine, ie. at 9 months of age, a ≥4-fold rise in GMT of IgG-anti-FHA occurred in 24.1% of subjects, a decrease occurred in 44.9% of subjects, and 31.0% of subjects had FHA antibody levels which remained the same or increased only slightly.

The anti-PT GMT fell to a nadir at 4 months of age, rose at 6 months and then fell again at 9 months (ie. 3 months after the third dose). There was increase in anti-PT GMT after 3 doses of vaccine. 19% of subjects experienced a ≥4-fold rise in anti-PT level, 63.8% a fall and 17.2% had levels that remained the same or increased only slightly after 3 doses of DTP.

The 3 children with pertussis had a rapid and marked rise in levels of PT, FHA and AGG2,3 antibodies at the time of postulated infection (data in Chapter 9). Cord blood levels of anti-PT and anti-FHA were lower than the mean level in 2 cases and higher than the mean in the third case. A response to PT and to FHA did occur in the malnourished children, however peak antibody levels were usually lower than the mean of the well- nourished group (data given in Chapter 11).

Agglutinogens 2.3 (AGG2.3): IgG-antibody responses to W-DTP are shown in Table 7.5, Figure 7.4. There was a slight increase in GMT to AGG2,3 between pre- and post-vaccination sera. IgG anti-AGG2,3 levels fell from birth to 2 months and then rose with each dose of vaccine. Seroconversion rate (a ≥4-fold rise in level from pre-vaccination levels at 2 months of age) was 67.2%. Although the group as a whole had increased antibodies to AGG2,3, the response of individuals was absent or poor in approximately one third of cases. A decrease in GMT occurred in 16.4% of recipients and 16.4% had titres that remained the same or increased only slightly after 3 doses of vaccine.

A rapid rise in anti-AGG2,3 titre occurred at the time of infection in the 3 infants with pertussis infection. Peak anti-AGG2,3 levels in all malnourished children were lower than the mean level

in well-nourished infants.

7.6 DISCUSSION

The South African whooping cough vaccine has been reported to be very effective in the Cape (Metcalf *et al.*, 1989). In the present study this triple vaccine with a whole cell pertussis component appeared to be fairly safe. The infants were carefully monitored for complications and the rates of minor local and systemic post-vaccination events were low. Only 7.6% of subjects incurred a local or systemic symptom, whereas rates of 50% and 72% have been reported in some studies in developed countries (Cody *et al.*, 1981,1982; Barkin & Pichichero, 1979) and is suggestive of a low potency vaccine or poor observation. Fever, the most frequently occurring post-vaccination event occurred at a rate of 32.1/1000 doses as compared with 297 and 254, respectively,in Japanese and Swedish studies of whole-cell vaccine (Sato *et al.*, 1984; Blennow *et al.*, 1986). Local symptoms, absent in the present study, occurred in 16.7% (Baraff *et al.*, 1984) to 72.2% (Barkin & Pichichero, 1979) of vaccinees in other studies. Cody *et al.* (1981) found that local reactions following whole-cell DTP were generally more frequent with increasing dose of vaccination, whereas systemic reactions, with the exception of fever, were less frequent. In this study, systemic reactions, with the exception of loss of appetite, were less frequent with each vaccine dose.

The South African DTP was associated temporally with major post-vaccination events (convulsions and hypotonic-hyporesponsive episodes) which did not appear to produce detectable sequelae. The incidence of convulsions with whole-cell DTP in the present study appeared high (1/249 doses, ie. 4.02/1000 doses) compared with other DTP vaccine studies in the United Kingdom (0.4/1000 doses) and the United States (0.57/1000 doses) (Cody *et al.*, 1981) but can only be expected to occur at any predictable level in study groups numbering $\geq$5000. In our studies of acellular pertussis (see Chapter 8) and measles vaccines among Black children in South Africa (unpublished observations) rates of convulsions were 1.7/1000 and

0/136 doses, respectively.

Convulsions occur in young children as a result of many aetiologies. Signs of underlying neurologic disorders may be brought out by the common systemic effects of DTP vaccination or any other fever-producing event. It has been estimated that by coincidence alone 0.7 infants per 1000 will have a convulsion due to other causes within 1 week of DTP vaccination. Hence no causal relationship could be established with W-DTP vaccination.

The hypotonic-hyporesponsive state appears to be a reaction unique to pertussis vaccination. A hypotonic-hyporesponsive episode occurred in 2 children within 7 days after immunisation (8.02/1000 doses). In a study in the United States, (Cody *et al.*, 1981) this type of reaction occurred at a rate of 0.57/1000 doses, while in our study of an acellular pertussis vaccine (see Chapter 8) this problem did not occur.

The small number of vaccinees in the present study did not allow statistical evaluation of the occurrence of alleged neurologic complications following W-DTP vaccination and hence prevents any conclusion about whether incidence is reduced by acellular vaccine administration.

The risk from major post-vaccination events cannot be fully quantified in a study of this size and duration, particularly as the study population had a high incidence of infectious diseases. There are no data on the incidence of these events in unvaccinated children. From the point of view of study design it would have been preferable to have included a randomized control group of children not receiving any pertussis vaccine but it was not ethically feasible to include such children in the study. The possibility of these reactions being due to other intercurrent phenomena cannot therefore be excluded.

PT, FHA and agglutinogens have been shown to be immunogenic constituents of whole-cell

vaccine (Ashworth *et al.*, 1983). In view of the reported efficacy of South African whole-cell DTP (Metcalf *et al.*, 1989) it was therefore both surprising and interesting that serum IgG antibody responses to FHA and PT, 2 major antigens of *B. pertussis* believed to be related to protective immunity, occurred infrequently and that no increase in GMT of either antibody was found after 3 doses of South African W-DTP vaccine. Serum antibody responses to these antigens have been reported in immunogenicity studies of whole cell vaccines elsewhere (Baraff *et al.*, 1984; Lewis *et al.*, 1986; Thomas *et al.*, 1985; Edwards *et al.*, 1986,1989). Although evidence for protection by anti-PT and anti-FHA antibodies has been obtained in mice (Sato *et al.*, 1984; Oda *et al.*, 1984; Sato & Sato, 1984) and serospecific protection correlated with serum agglutinin type has been reported (Rutter *et al.*, 1988; Ashworth *et al.*, 1983) for this species no serological correlates of protection against pertussis infection have yet been discovered for humans. No one class or type of pertussis agglutinin antibody has been demonstrated to be protective in humans and it is unknown whether antibodies to agglutinogens alone are sufficient to confer immunity (Pichichero *et al.*, 1987). Furthermore, the acquisition of IgG antibodies to PT and FHA have not been shown to correlate to vaccine efficacy or to clinical immunity.

Because of the lack of responses to FHA and PT in the vaccinees, the high levels of all 3 antibodies found in maternal and cord sera suggest a high prevalence of infection amongst women of child-bearing age as well as active transplacental transfer of antibody. All the maternal sera had substantial levels of antibodies to FHA and to AGG2,3 and many also had similar levels of anti-PT. However, there was considerable variation and this was reflected in the cord blood antibody levels. The poor response to vaccination may have been related, in part, to the 'dampening' effect of maternally-acquired antibodies. Further investigation of this phenomenon has been discussed in Chapter 10.

Geometric mean titres rather than seroconversion have been emphasized in the assessment of serological responses since poor seroconversion in those with high maternal titres are a

reflection of initial antibody levels and do not indicate impaired responsiveness.

For one infant with suspected whooping cough, cord blood levels of all 3 antibodies were higher than the mean cord blood levels, whereas for the other 2 suspected cases they were lower. The malnourished children overall responded no less well to vaccination than did other vaccinees. Nevertheless, the immune response was delayed in some cases and further study of pertussis vaccination of malnourished children is needed.

This study raises serious questions about the South African whooping cough vaccine which need to be resolved. Most importantly a wider study of vaccine efficacy coupled with serological monitoring is required to determine whether the results presented here are typical or if they arise from batch-to-batch variability of the vaccine. The poor immunogenicity of the whole-cell DTP used in this study, at least with regard to FHA and PT, signifies either -

i. this batch would not provide protection, contrary to expectations based on reported efficacy; or

ii. any protection was likely to be due to some vaccine constituent(s) other than PT or FHA.

At present the benefits of vaccination with the vaccine appear to outweigh the disadvantages (Hinman & Koplan, 1984) therefore despite the reservations expressed here, there is no indication for withdrawal of the vaccine and current vaccination programmes should be encouraged.

TABLE 7.1 Incidence of intercurrent illnesses in recipients of whole-cell pertussis vaccine from birth to 9 months of age.

TYPE OF ILLNESS	NO OF ILLNESSES AGE (MONTHS) 0≤2 N=91	>2≤4 N=83	>4≤6 N=75	>6≤9 N=60	TOTAL (% OF TOTAL ILLNESSES)
Upper respiratory tract infection	20	18	14	12	64(37.2)
Skin infection	13	13	15	11	52(30.2)
Diarrhoea	1	6	5	2	14(8.1)
Lower respiratory tract infection	4	4	3	1	12(7.0)
Eye infection	1	2	1	2	6(3.5)
Measles	0	0	0	2	2(1.2)
Hepatosplenomegaly	2	3	1	0	6(3.5)
Viral meningitis	0	0	1	0	1(0.6)
Jaundice	1	0	0	0	1(0.6)
Pyrexia of unknown origin	1	2	0	0	3(1.7)
CNS-related*	3	2	1	0	6(3.5)
Chicken pox	0	0	0	1	1(0.6)
Oral herpes	0	0	0	2	2(1.2)
TOTAL NUMBER OF ILLNESSES	47	50	42	33	172
PERCENTAGE OF ILLNESS**	27.3	28.5	25.0	19.2	100

* Craniotabes, Bossing, Large fontanelle

** Total number of infants with ≥ 1 illness = 70

TABLE 7.2 Dose-related incidence of post vaccination events (pve) following administration of whole-cell pertussis vaccine in very young african infants.

POST-VACCINATION EVENT	INCIDENCE/1000 DOSES (n) DOSE 1 (n=91)	DOSE 2 (n=83)	DOSE 3 (n=75)	TOTAL (n=249)
Major events				
Hypotonic-hyporesponsive episodes	22.0(2)	0	0	8.0(2)
Convulsions	0	0	13.3(1)	4.0(1)
Systemic events				
Fever	44.0(4)	36.1(3)	13.3(1)	32.1(8)
Excessive crying	54.9(5)	12.0(1)	0	24.1(6)
Loss of appetite	0	0	0	8.0(2)
Irritability	11.0(1)	0	0	4.0(1)
Local events (at injection site)				
Swelling	0	0	0	0
Induration	0	0	0	0
ALL	131.9(12)	48.2(4)	40.0(3)	76.3(19)

TABLE 7.3 Post-vaccination events in recipients of whole-cell pertussis vaccine.

SUBJECT No	SYMPTOM DOSE 1	DOSE 2	DOSE 3	TOTAL
1	F,H,C	nil	nil	3
2	F,C,I	nil	nil	3
3	nil	F	F	2
4	nil	F,C	nil	2
5	nil	F	nil	1
6	F	nil	nil	1
7	C	nil	nil	1
8	C	nil	nil	1
9	F	nil	nil	1
10	nil	nil	Co	1
11	H	nil	nil	1
12	nil	nil	L	1
13	C	nil	nil	1
TOTAL	12	4	3	19

Key C = crying
Co = convulsion
F = fever
H = hypotonic-hyporesponsive episode
L = loss of appetite

TABLE 7.4 Maternal and cord blood levels (GMT ± SE)* of pertussis IgG antibodies in recipients of whole-cell pertussis vaccine (range).

ANTIBODY	MATERNAL	CORD
Anti-PT	41.6 ± 6.5 (2.3-211.4) (N = 55)	48.2 ± 9.8 (1.4-466.6) (N = 56)
Anti-FHA	72.2 ± 12.1 (12.3-590.9) (N = 56)	78.3 ± 15.1 (5.3-595.4) (N = 55)
Anti-AGG2,3	418.37 ± 210.1 (16.2-11603.1) (N = 55)	539.9 ± 300.2 (0.6-14166.2) (N = 54)

* Geometric mean titre ± standard error.

TABLE 7.5 Serologic responses to whole-cell pertussis vaccination in African infants.

AGE (months)	SAMPLING TIME	GMT ± SE*: ELISA U/ml (RANGE) Anti-FHA	Anti-PT	Anti-AGG2,3
2	Before vaccination	82.8 ± 46.0 (1.6-135.1) N = 50	31.7 ± 10.3 (1.0-483.9) N = 49	212.5 ± 98.0 (6.35-4246.4) N = 48
4	Eight weeks after Dose 1	68.3 ± 20.0 (0.9-325.9) N = 54	20.5 ± 6.2 (0.9-310.8) N = 54	359.1 ± 148.53 (0.6-6556.4) N = 50
6	Eight weeks after Dose 2	62.5 ± 14.9 (0.9-255.6) N = 60	23.4 ± 6.9 (0.6-4453.9) N = 57	503.2 ± 114.5 (0.9-290.0) N = 54
9	Twelve weeks after Dose 3	60.8 ± 12.5 (0.9-183.6) N = 50	18.4 ± 4.2 (1.2-186.2) N = 45	738.1 ± 165.3 (0.6-5978.7) N = 44

* Geometric mean titre ± standard error.

SUBJECT AGE	VACCINATION	CLINICAL ASSESSMENT AND BLOOD SAMPLING
8-12 weeks	W-DTP[1], TOPV[2]	+
4 months +/-2 weeks	W-DTP, TOPV	+
6 months +/-2 weeks	W-DTP, TOPV	+
9 months +/-2 weeks	measles	+

1 Whole-cell diphtheria-tetanus-pertussis.

2 Trivalent oral polion vaccine.

Figure 7.1 Study design.

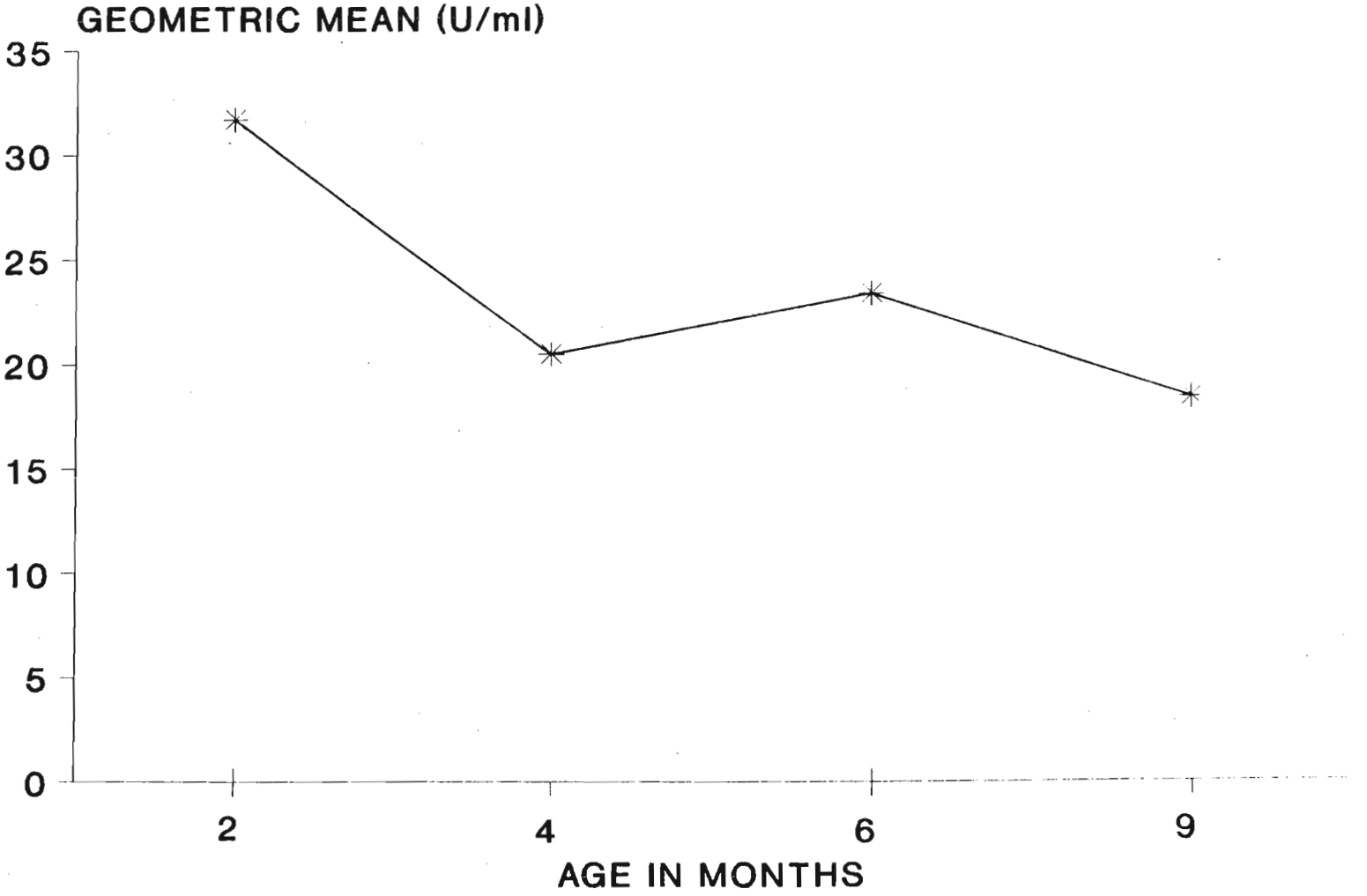


Figure 7.2 IgG-PT responses to whole-cell pertussis vaccination.

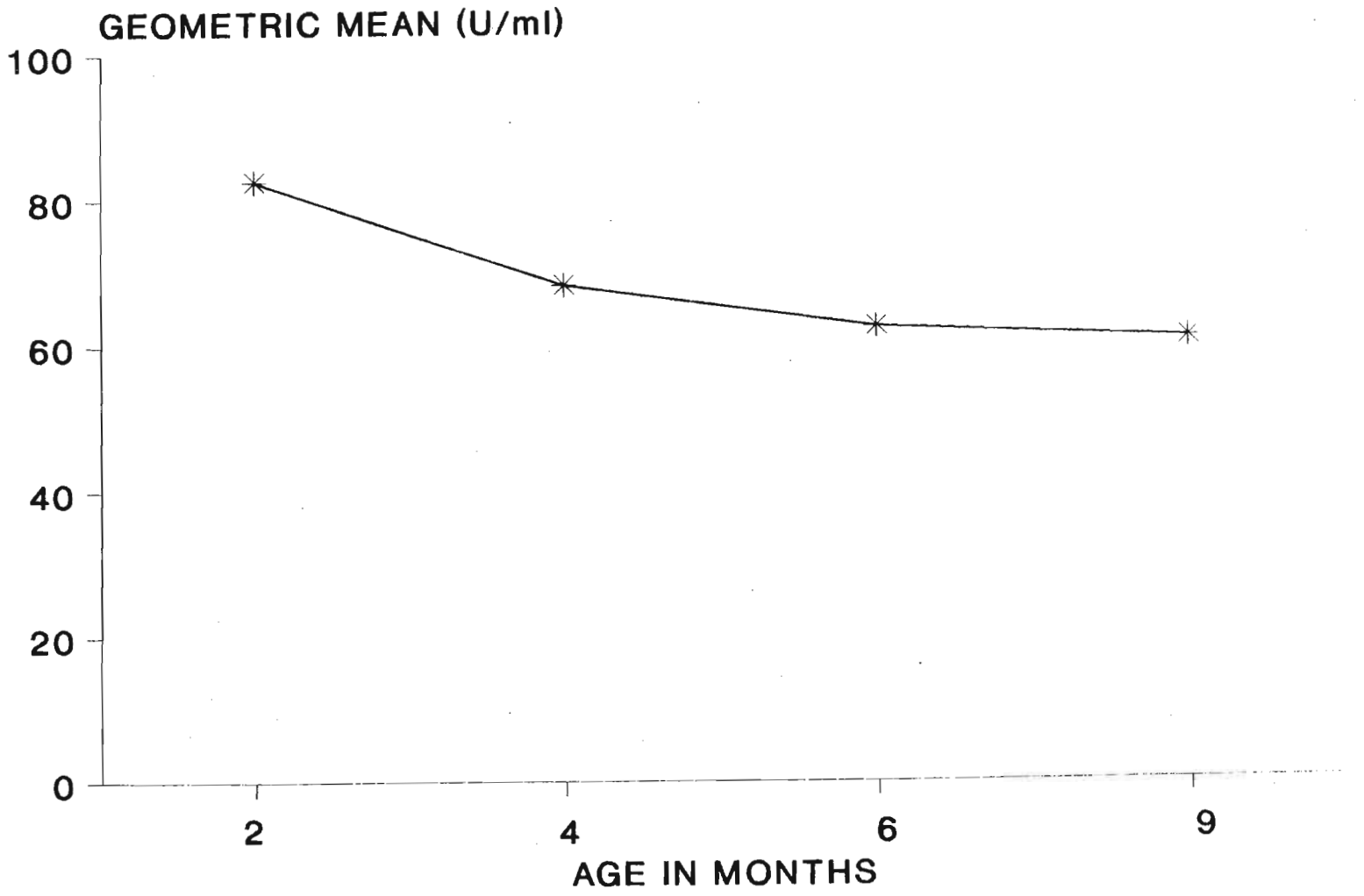


Figure 7.3 IgG-FHA responses to whole-cell pertussis vaccination.

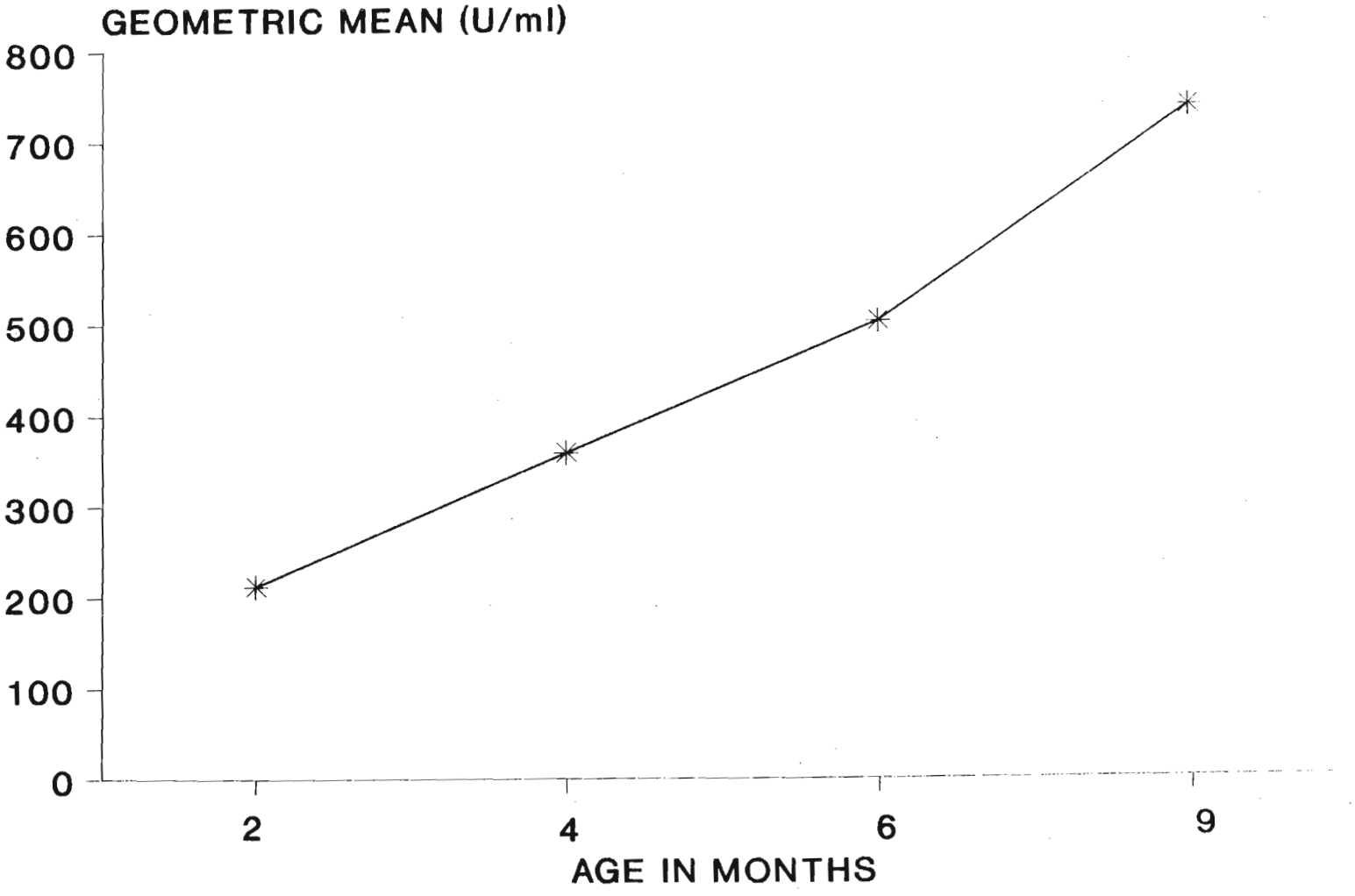


Figure 7.4 IgG-AGG2,3 responses to whole-cell pertussis vaccination.

CHAPTER 8

IMMUNOGENICITY AND POST-VACCINATION EVENTS FOLLOWING ACELLULAR AND WHOLE-CELL PERTUSSIS VACCINES IN VERY YOUNG AFRICAN BABIES VACCINATED ROUTINELY AND AT BIRTH

8.1 OBJECTIVES

The objectives of the comparison between Japanese acellular pertussis vaccine containing pertussis toxin (PT) and filamentous haemagglutinin (FHA) in a 1:1 ratio combined with diphtheria (D) and tetanus (T) toxoids and the South African conventional combined trivalent DT-whole-cell pertussis vaccine were -

i. To compare the short-term safety and reactogenicity of the pertussis component of the 2 vaccines from the beginning of the routine primary vaccination course (month 2) until the age of 9 months. This was assessed by recording all medical events with special attention to neurologic symptoms and systemic infections.

ii. To compare the serologic responses to the pertussis component of the 2 vaccines after routine primary vaccination by measuring specific IgG antibody responses to individual *B. pertussis* antigens; PT, FHA and agglutinogens 2 and 3 (AGG2,3) in serum samples collected before, during and after the vaccination course.

iii. To evaluate the immunogenicity and safety of neonatal vaccination with acellular pertussis vaccine.

iv. To compare serologic responses following 4 versus 3 doses of acellular pertussis vaccine.

8.2 SUMMARY

Acellular pertussis vaccine has not been evaluated previously in neonates. The effect of neonatal vaccination with acellular pertussis vaccine on subsequent immunity; and the immunogenicity and short-term safety of routine primary vaccination with this vaccine compared with conventional whole-cell preparations was investigated in a phase II trial.

Three hundred and forty-five healthy, full-term African babies were enrolled in the study at birth; 58% of whom were successfully followed for 9 months. Infants were assigned to 1 of 3 vaccine groups in sequence at birth and received either acellular or whole-cell pertussis (P) vaccine combined with D and T as DTP, at 2, 4 and 6 months of age. Groups I and II received acellular-DTP (A-DTP) and Group III whole-cell (W-DTP). In addition, at birth, Group I received an additional dose of A-DTP and Group II, a saline placebo injection. No unvaccinated controls were studied for ethical reasons.

Serologic IgG responses to 3 major protective antigens of *B. pertussis*, FHA, PT and AGG2,3 were measured by ELISA in sera obtained at birth, and prior to vaccination at 2, 4 and 6 months and at 9 months of age. The incidence and nature of post-vaccination events were recorded for 14 days after each dose.

A-DTP induced serum IgG responses to PT and FHA comparable with those reported in other studies, with peak PT titres occurring at 6 months of age after 2 doses in babies vaccinated according to the routine schedule, ie. from 2 months of age (Group II). Response to W-DTP was found merely to restore levels of antibody to PT and FHA to those found in cord blood after 3 doses of vaccine, though high levels of IgG-AGG2,3 were produced. Significantly lower final AGG2,3 titres ($p = 0.0001$) were recorded in recipients of A-DTP.

In Groups I, II and III respectively, the anti-PT seroconversion rates were 54.2%, 75.4% and 19.0%; the anti-FHA rates were 23.3%, 69.2% and 24.1% and the anti-AGG2,3 rates were 22.6%, 22.1% and 67.2%.

Four doses of A-DTP (Group I) did not produce higher seroconversion rates or titres than the 3-dose schedule. Substantial titres of all 3 antibodies measured in cord blood were indicative of active transplacental transfer.

Incidence of all post-vaccination events to both acellular and whole-cell vaccines was low, (85.96/1000 doses, 136.36/1000 doses and 76.30/1000 doses in Groups I, II and III respectively). Minor symptoms were more common in recipients of acellular vaccine, although no significant differences in rates were demonstrated. Neurologic post-vaccination events (without sequelae) occurred more frequently in recipients of whole-cell vaccine (Group III); hypotonic-hyporesponsive episodes and convulsions occurred at rates of 8.03/1000 doses and 4.02/1000 doses respectively. One infant who received a neonatal dose of A-DTP (Group I) experienced a convulsion after the second injection at 2 months of age (1.69/1000 doses). No infant vaccinated according to the routine schedule with A-DTP (Group II) experienced a neurologic symptom.

Eleven infants with pertussis infection, (10 subclinical) were diagnosed retrospectively on the basis of serologic evidence. Most of the subjects were unvaccinated; only 1 had completed the primary vaccination course, and all were 8 months of age or younger.

A-DTP vaccination commencing at birth produced final antibody titres and seroconversion rates to PT and FHA which were superior to those of South African whole-cell vaccine but were considerably lower than when the vaccine was incorporated into routine schedules commencing at 2 months of age. The study findings suggest that acellular pertussis vaccines, whether given from birth or from the age of 2 months, appeared safer and produced a better serologic response than the South African whole-cell product which has impaired immunogenicity. Further and more extensive studies are indicated before acellular vaccines are incorporated into routine vaccination schedules.

8.3 INTRODUCTION

Pertussis (whooping cough) is still a major life-threatening disease in the Third World,

estimated by WHO to cause the death of more than 60,000 children in Africa and 500,000 children worldwide each year (WHO, 1988); virtually all in unvaccinated infants. Pertussis is not a notifiable disease in South Africa and there is consequently a paucity of hard data on efficacy and few cases are proven. Some hospital-derived evidence (see Chapter 6) suggests that the incidence, morbidity and mortality of the disease are highest, as elsewhere in infancy; the disease is especially severe in those less than 6 months of age (Annual summary, 1980). Vaccination with effective vaccines soon after birth can not only give early protection but also improve vaccine coverage rates.

The conventional vaccine against pertussis contains killed whole-cells of *B. pertussis* and is associated with a number of well-recognised adverse effects, ranging from mild local and systemic reactions to severe reactions resulting in neurologic sequelae. The exact incidence and causal relationship of the latter to pertussis vaccination has not been conclusively established.

The number of children vaccinated with whole-cell pertussis vaccine has declined in several developed countries during the past decade because of parental concern about the high rate of vaccine-associated reactions. Despite these concerns, various studies have concluded that the benefits of conventional whole-cell pertussis vaccination far outweigh the risks, more especially in the Third World (Koplan *et al.*, 1979; Hinman & Koplan, 1984; Miller *et al.*, 1982); and the WHO continues to recommend primary whole-cell DTP vaccination at 2, 4 and 6 months of age.

A purified pertussis vaccine containing the currently marketed D and T components (aluminium-phosphate-adsorbed) in combination with 2 acellular pertussis antigens, PT and FHA was first used in Japan in 1981 and has since been used for mass vaccination of over 40 million Japanese children over 2 years of age (Sato *et al.*, 1984; Kimura & Kuno-Sakai, 1990).

Clinical and serological data from Japan and Sweden have shown acellular vaccines to be

effective and to be associated with fewer adverse effects than conventional whole-cell pertussis vaccines (Edwards *et al.*, 1986; Lewis *et al.*, 1986). Existing data pertaining to the immunogenicity and safety of acellular vaccines relates largely to infants aged 2 years or more. The response to these vaccines given at 2-3 months, the age at which primary routine vaccination commences, remains to be resolved. In addition there is no conclusive evidence that the vaccine is associated with fewer neurologic events, hence the reluctance of most authorities to recommend their immediate introduction for use in infancy.

In order to address the problem of high morbidity and mortality from pertussis in early infancy; and the incorporation of a new and apparently safer vaccine into routine vaccination schedules, a phase II trial of acellular and whole-cell pertussis vaccines was undertaken in very young infants.

8.4 STUDY DESIGN

Study design is shown in Figure 8.1. This was an open-unblinded study. Vaccines were given as a primary course of 3 or 4 consecutive doses commencing either at birth or at 2 months of age, and at 8-12 week intervals thereafter. Subjects were assigned to one of three groups of 115 each in sequence at birth. At birth Group I received A-DTP; Group II received a saline placebo and Group III received no vaccine. At 2, 4 and 6 months of age Groups I and II received A-DTP, and Group III received W-DTP.

Parents and nursing staff were unaware of which vaccine was given. Local and systemic post-vaccination symptoms were recorded for 14 days after each dose. Each child was weighed and examined by a paediatrician prior to vaccination at every clinic visit and blood samples were collected at this time. Furthermore, infants were monitored for intercurrent illnesses up to 9 months of age, with special attention being paid to nutritional state, common childhood infections, and whooping cough symptoms. The study was approved by the Ethical Committee

of the Faculty of Medicine, University of Natal. No unvaccinated controls were studied for ethical reasons.

In addition, in accordance with WHO recommendations, infants received BCG and TOPV at birth; TOPV at 2, 4 and 6 months, and measles vaccine at 9 months.

8.5 MATERIALS AND METHODS

Details of patients and methods are provided in Chapter 5, hence a very brief account is given here in this section so that this chapter can be read as a whole.

8.5.1 SUBJECTS

Selection, recruitment, follow-up: In the 3 month period from March to May 1988, 345 healthy full-term newborn male and female infants from Kwa Mashu, a suburb of Durban, South Africa inhabited exclusively by blacks, were enrolled in the study after written informed consent was obtained from the parents or guardians of the child. Anthropometric data and details of family circumstances were obtained from the mother at this time.

Three deaths which were not vaccine-related occurred before 2 months of age. All 3 children had been assigned to the whole-cell vaccine group (Group III) and had therefore received only BCG and TOPV at birth. Of the 342 remaining subjects, 285 (83%) returned at 2 months of age, 256 (75%) at 4 months, 232 (68%) at 6 months and 198 (58%) at 9 months of age. There was no significant difference between groups in the loss of subjects.

Exclusion criteria: Children were excluded if any of the following were present: Any form of acute illness or incubation phase of an illness at the time of vaccination; simultaneous participation in any other clinical study; planned moving-out of the study area within the study period; any form of congenital disease, malformation or serious chronic disorder (ie. Down's

Syndrome, diabetes, etc.); premature infants or infants from high-risk deliveries or pregnancies or infants with an abnormal birth history.

Contraindications for the following vaccine dose: Pronounced reaction after a previous dose, ie. serious reactions as described in Section 8.5.4; seizures, abnormally high temperature, persistent crying ≥ 3 hours, collapse or shock-like reaction within 48 hours after a dose.

8.5.2 ETHICAL JUSTIFICATIONS FOR THE STUDY

The majority of infants in the study received an acellular vaccine for primary vaccination instead of the current whole cell preparation. This was considered ethically justifiable since data already available suggests that acellular vaccines are immunogenic and are as safe as conventionally used pertussis vaccines.

Ethical Committee: The study was approved by the Ethical Committee of the Faculty of Medicine, University of Natal. Local health authorities were notified about the conduct and nature of the study.

Informed Consent: The investigators or study nurse informed parents or guardians of the aims and possible side effects of the research trial in their native language (Zulu), prior to enrollment and their written consent was obtained. The Subject Information Sheet is shown in Appendix 2.

Withdrawals: Subjects were free to withdraw from the study for any reason at any time; the reasons were recorded by the investigator. Dropouts were not replaced.

8.5.3 NUTRITIONAL STATUS

Nutritional status of subjects was assessed at birth and at each clinic visit by the anthropometric indices of length and weight for age; and clinical features of protein-energy malnutrition (PEM), vitamin or trace-element deficiencies. The National Centre for Health

Statistics Reference Population was used as a standard (NCHS, 1976). Subjects with a weight-for-age less than the third percentile were considered underweight, and children with a length-for age less than 90% of the median stunted (Waterlow *et al.*, 1977).

8.5.4 EVALUATION AND REPORTING OF POST-VACCINATION EVENTS

Parents recorded the presence of local symptoms (swelling or induration of any size) at the injection site, systemic symptoms (clinical impression of fever, excessive crying, fretfulness, appetite change, hypotonic-hyporesponsive episodes, convulsions or other neurologic events) or other symptoms regarded as possibly vaccine-associated on a specially designed illustrated record sheet (Appendix 1) for 14 days after each dose. Quantitative measurements of fever and diameters of induration or swelling were not made (as reported elsewhere) because of inadequate facilities and limited educational background of parents. Detailed instructions pertaining to understanding of the record sheet, especially with regard to convulsions and hypotonic-hyporesponsive episodes, were conveyed in Zulu by a registered nurse. At each clinic visit parents were questioned about any untoward effects that had followed the previous injection.

Information pertaining to the vaccine trial as well as contact addresses and telephone numbers of investigators in Zulu and English (Appendix 2 and 3) were attached to each subjects' vaccination record chart.

In addition, parents were instructed to contact the study nurse or one of the investigators immediately in case of one of the following -

i. Suspicion of whooping cough disease (persistent cough lasting for 7 days or more, with or without associated whoop or vomiting). These criteria are the same as those used by the WHO in clinical trials of acellular pertussis vaccines in Sweden. (*Ad Hoc* Group for the Study of Pertussis Vaccines, 1988).

ii. Neurological reactions such as convulsions, collapse and/or unexplained loss of

consciousness, infantile spasms.

iii. General allergic reactions.

iv. Any serious reaction of unforeseen nature where the child's life is endangered.

v. Severe local reaction (pain, induration or swelling) at the injection site.

vi. Any other sign or symptom that caused the child's attendants to seek medical assistance.

Results were reported as follows -

i. Post-vaccination symptoms / n doses.

ii. Number of infants with post-vaccination event.

8.5.5 BLOOD SAMPLING

Sera for antibody assays (3-5 ml) were obtained at birth from mothers' blood and cord blood, and from whole blood taken immediately prior to DTP vaccination at 2, 4 and 6 months and at 9 months of age at the time of measles vaccination. Sera were coded and frozen at -20°C until assays could be performed. All sera from one individual were tested in the same assays on the same day. In some cases the sample was not sufficient to carry out all the tests required and therefore the number of samples giving rise to the data shown in the tables are not uniform.

8.5.6 VACCINES AND VACCINE ADMINISTRATION

Acellular DTP vaccine: The acellular vaccine employed in the study was the Biken B-type (J-NIH-6) combined, purified trivalent diphtheria - tetanus-acellular pertussis vaccine (aluminium-phosphate adsorbed) preserved with 0.01% w/v ie. <0.05 mg thiomersal.

The vaccine contained 28 Lf units diphtheria toxoid; 7 Lf units tetanus toxoid, 0.075 mg aluminium phosphate, as an adjuvant in phosphate-buffered saline (PBS); and more than 4 IU purified pertussis antigen (PT and FHA) per 0.5 ml dose. It was supplied as a single lot (Lot 21B) by the Kanonji Institute, Research Foundation for Microbial Diseases (BIKEN) of Osaka

University, Japan and the Japanese National Institute of Health (JNIH).

The total PT content of the vaccine was 7.5 mg/l protein nitrogen, the total FHA content 7.5 mg/l protein nitrogen and the total protein content 0.18 g/l. Documentation on tests of content and purity of the vaccine is contained in Appendix 7. The package insert supplied by the manufacturer is shown in Appendix 8.

Whole-cell DTP vaccine: The South African conventional whole-cell pertussis vaccine employed in the study was a combined trivalent diphtheria-tetanus-whole-cell pertussis aluminium-phosphate adsorbed preparation. The vaccine contained 25 Lf units diphtheria toxoid, 6 Lf units tetanus toxoid, 10,000 $x10^6$ *B. pertussis* organisms, 1.25 mg aluminium phosphate per and 0.01% (w/v) thiomersal per 0.5 ml dose. It was supplied as a single lot (Lot A595) by the South African Institute for Medical Research (SAIMR) Johannesburg, South Africa. Date of manufacture - 01.01.88. Date of expiry - 01.01.90. The package insert supplied by the manufacturer is shown in Appendix 9.

Both vaccines were injected into the left anterolateral thigh in 0.5 ml doses. Before each injection the skin at the injection site was thoroughly cleansed with an alcohol swab. Saline placebo and A-DTP were administered via the subcutaneous route; and W-DTP via the intramuscular route. All vaccines were stored in the refrigerator (4°C) and proper cold chain conditions were maintained throughout.

8.5.7 SEROLOGIC ASSAYS

Serum samples were assayed for determination of antibody titres to specific components of the *B. pertussis* organism with potential protective properties, namely FHA, PT and AGG2,3. IgG antibodies to FHA, PT and AGG2,3 were assayed by ELISA at the Centre for Applied Microbiology and Research, Public Health Laboratory Service, Porton, UK by the author. The ELISA procedure used was essentially as described by Rutter *et al.* (1988). Optimal conditions

for ELISA were determined by chequerboard titrations. Results were expressed as ELISA units per ml (U/ml). Serum antibody responses were assessed for each antigen by comparing -

i. The difference in geometric mean titre (GMT) for each group between pre- and post-vaccination sera.

ii. The percentage in each group with seroconversion, ie. ≥4-fold rise in antibody titre between pre-vaccination sera (cord blood for Group I, and 2-month samples for Groups II and III) and post-vaccination sera.

Reference Sera: Japanese National Institute of Health (JNIH) Reference Standard Human IgG Pertussis Serum positive for antibodies to PT, FHA and AGG2,3. Lot 10, supplied by Biken-Kanonji Institute (Osaka, Japan) as a freeze-dried preparation containing 250 ELISA U/ml PT antibody and 400 ELISA U/ml FHA antibody, was used throughout to allow calculation of antibody unitage. This sera was assigned a value of 400 ELISA U/ml of IgG-anti-AGG2,3. The optical densities of the reference serum dilution series on each plate was subject to logistic analysis. The titre of each test serum relative to the reference serum on the same plate was calculated by means of parallel line assays.

Antigens: Purified pertussis FHA and PT (50% glycerinated) each containing 200 μg protein Nitrogen/ml were supplied by the Biken-Kanonji Institute for use in ELISA. (These products were the same as those sent to the National Bacteriological Laboratory of Sweden for the WHO Collaborative Study). The fimbrial antigen, co-purified AGG2,3 containing 500 μg/ml protein Nitrogen was supplied by Dr. A. Robinson, Biologics Division, PHLS, CAMR, Porton UK as a freeze-dried preparation containing AGG2,3 in a ratio of 6:4.

Assay Procedure: Details are elucidated in Chapter 5.

8.5.8 STATISTICAL ANALYSIS

Subjects: The drop-out rate per group was compared by chi-square tests.

Serology: All serologic results were converted to geometric values before analysis. Paired Student's 't' test with 2-tailed probability was used for comparison of pre- vs. post-vaccine GMT's. Group comparisons of GMT's were made using Duncans' Multiple Range Test which controls the type I comparison error rate, or the unpaired Student's 't' test with 2-tailed probability for statistical significance ($p < 0.05$ was considered a statistically significant difference on a 95% confidence level). Seroconversion rates were compared using Fisher's exact test. Subjects with suspected pertussis infection were excluded from analysis of serologic responses.

Post-vaccination events: Comparison of clinical reaction rates in the 3 vaccine groups were made using 2x2 chi square contingency tables with each post-vaccination event counted as present or absent at any time during each 14-day follow-up period. $P < 0.05$ was considered a statistically significance difference on a 95% confidence level.

8.6 RESULTS

8.6.1 PERTUSSIS INFECTION

Eleven cases of whooping cough infection occurred during the study period, 10 of which were subclinical (see Chapter 9). Diagnosis was retrospective and based on a rapid and marked ($\geq$ 4-fold) increase in levels of IgG antibodies to AGG2,3 and to either or both PT and FHA between 2 consecutive sera at the time of postulated infection, followed by a rapid decline in levels. Diagnosis was confirmed by the detection of IgA antibodies (data not included) which appear after infection but not after vaccination (Winsnes *et al.*, 1988). To eliminate the possibility of inadvertent vaccine administration, ELISA for D and T antibodies were performed.

One case occurred in Group I, 7 in Group II and 3 in Group III. All cases occurred in infants 8 months of age or younger. Only 1 infant (Group III) had completed primary vaccination. Four

infants were unvaccinated (2 months of age), 5 had received 1 dose of vaccine (4 A-DTP, 1 W-DTP), and 1 had received 2 doses of A-DTP). Clinical signs of pertussis infection were absent in all but one 4-month old infant (Group II) who presented with subconjunctival haemorrhage and cough of more than 1 weeks' duration. This infant did not experience subsequent post-vaccination symptoms. Four infants were reported to have had upper respiratory tract infections around the time of subclinical infection which may have been subclinical or modified pertussis.

The infant from Group I was underweight at birth (at the time of first A-DTP vaccination) and subsequently developed clinical disease at 4 months of age after 2 doses of vaccine. Other clinical problems experienced by this infant were anaemia and skin infection at 2 months and diarrhoea at 6 months.

8.6.2 CLINICAL RECORD

Details of intercurrent illnesses in infants from birth to 9 months of age are shown in Table 8.1, 8.2 and 8.3. The overall incidence of infections, intercurrent illnesses or clinical signs thereof during the study period was similar in all vaccine groups. During the first 9 months of life 66 infants in Group I had 159 illnesses; 70 infants in Group II had 167 illnesses, and 70 infant in Group III had 172 illnesses. For all infants a total of 498 infections, illnesses or clinical signs thereof were detected, the highest incidence of which occurred between 6 and 9 months of age (36.7% of all illnesses). The number of illnesses between ((0<2 months and between 6 and 9 months, was significantly higher in whole-cell vaccines (Group III) ($p < 0.0001$). Infections of the upper respiratory tract were the most commonly occurring (35.5% of all illness), followed by skin infections (26.9%) and diarrhoea (11.0%).

8.6.3 NUTRITIONAL STATUS

The 3 vaccine groups were similar on enrollment with respect to length and weight. A total of 25 infants with PEM (small-for-gestational age or post-natal) were diagnosed during the study

period. Antibody responses were not found to be affected by nutritional status (see Chapter 11).

8.6.4 POST-VACCINATION EVENTS

Parental evaluation of events occurring in the 14 days following each vaccination were derived from questionnaires returned by 591 dose-recipients of acellular DTP vaccine and 249 whole-cell DTP dose-recipients. Incidence of post-vaccination events following administration of acellular or whole-cell pertussis vaccines (doses 1, 2, 3 ± 4 combined) are shown in Tables 8.4 and 8.5.

The overall incidence/number of doses of all reactions to the acellular vaccine was low and not significantly different from that of the whole-cell vaccine with which it was compared.

Subject details with regard to A-DTP post-vaccination events are given in Table 8.6 and 8.7. Subject details with regard to W-DTP are given in Chapter 7 (Table 7.3). Twelve infants in Group I experienced a total of 30 post-vaccination events; 4 had 1 symptom, 5 had 2 symptoms, 2 had 5 symptoms and 1 had 6 symptoms. Twenty-one infants in Group II experienced a total of 33 post-vaccination events. Of these 12 had 1 symptom, 6 had 2, and 3 had 3 symptoms. Thirteen infants in Group III experienced 19 post-vaccination events. Nine had 1 symptom, 2 had 2 symptoms and 2 had 3 symptoms. Age-related incidence of post-vaccination events following acellular and whole-cell pertussis vaccines (per 1000 doses) are shown in Figure 8.2.

W-DTP recipients incurred more post-vaccination symptoms after the first dose than after subsequent doses, as did A-DTP recipients in Group II. Infants vaccinated from birth (Group I) experienced more vaccine-associated symptoms after the second dose ($p > 0.05$). Dose-related incidence of post-vaccination events in recipients of acellular and whole-cell pertussis vaccine are shown in Tables 8.8, 8.9 and 8.10 and Figure 8.3.

Major events:

i. Group I. One infant experienced convulsions, fever, excessive crying, induration at injection site, and loss of appetite within a week after the second dose of A-DTP at 2 months of age. The child was found to be normal on clinical examination at 4 months of age but was nevertheless given only DT vaccine subsequently.

ii. Group II. No infant experienced a neurologically associated post-vaccination event.

iii. Group III. One infant experienced a febrile convulsion and cough with wheeze within a week after the third dose of W-DTP. He was admitted to hospital 30 days later with measles and bronchopneumonia. Two other infants in this group experienced hypotonic-hyporesponsive episodes within a week after the first dose of vaccine. Hypotonic-hyporesponsive episodes and convulsions hence occurred more frequently in whole-cell vaccinees. No statistical difference in rates was observed. These subjects all appeared clinically normal on subsequent clinical examination.

Minor events: Fever and excessive crying were the most frequently occurring systemic symptoms following both acellular and whole-cell vaccines. Minor local and systemic symptoms occurred less frequently in Group I (85.96/1000 doses) than in Group II (136.36/1000 doses). Loss of appetite (0.9 vs. 0.4% respectively), excessive crying (4.3 vs. 2.4%), irritability (1.5 vs. 0.4%), swelling (0.3 vs. 0%) and induration (0.52 vs. 0%) were observed to occur more frequently in those receiving A-DTP than in those receiving W-DTP. Statistical differences in rates of minor symptoms were not demonstrated.

No recipient of W-DTP or saline placebo reported swelling or induration at the injection site. Two recipients of A-DTP experienced swelling at the injection site and 3 experienced an induration.

8.6.5 TRANSPLACENTAL TRANSFER OF PERTUSSIS IgG ANTIBODIES

Antibodies to PT, FHA and AGG2,3 were present in maternal sera and often detected in higher quantities in cord sera; indicating active transplacental transfer (see Chapter 10). Cord IgG anti-FHA and anti-PT levels were similar in neonates of all 3 vaccine groups. Statistical analysis of geometric mean pertussis antibody titres, Group I vs. Group II vs. Group III are shown in Table 8.11. There were no statistically significant differences in the pre-vaccination PT and FHA GMT's for any group. In Group III anomalously (but not significantly) high IgG-FHA at 2 months and IgG-AGG2,3 in cord blood may represent some unconscious bias in the grouping of subjects.

8.6.6 SEROLOGIC RESPONSES TO VACCINATION

Geometric Mean Titres (GMTs):

1. GMTs (ELISA U/ml) of IgG antibodies to PT, FHA and AGG2,3 in cord sera and at 2, 4, 6 and 9 months of age are presented in Figures 8.4 to 8.9 and Tables 8.12 to 8.14.
2. Statistical analyses of the change in GMT between birth and 4, 6 and 9 months of age are shown in Table 8.15.
3. Antibody responses to PT and FHA were significantly higher after A-DTP than after W-DTP.
4. Mean log titres achieved after the second dose of A-DTP were higher than after third (final) dose of W-DTP.

PT/FHA:

Groups I and II.

i. Peak anti-PT titres occurred at 6 months of age, higher levels were attained in Group II.
ii. Peak and anti-FHA titres occurred at 6 and at 9 months of age in Groups I and II respectively.
iii. In Groups I and II, post-vaccination PT and FHA antibody titres at 6 months of age were significantly higher than pre-vaccination titres ($p < 0.005$).
iv. In Group I, the anti-PT GMT (but not the anti-FHA GMT) increased significantly

($p = 0.0001$) between 2 and 4 months of age after the second dose of A-DTP.

v. In Group II, both anti-PT and anti-FHA GMTs decreased significantly ($p = 0.014$; $p = 0.0001$) respectively from birth to 2 months of age, then increased significantly after each dose ($p = 0.0001$) to peak at 6 months of age.

Group III

i. There was a significant decrease in GMTs of PT and FHA antibody between peak and final bleeds and pre-vaccination bleeds, ie. after 3 doses of W-DTP ($p = 0.005$).

ii. No significant increases in anti-PT and anti-FHA GMTs were noted between any vaccination.

iii. At 4, 6 and 9 months the anti-PT GMT was significantly lower compared with Groups I and II ($p < 0.001$).

AGG2,3:

Groups I and II

i. AGG2,3 antibody titres dropped significantly from birth to 2 months ($p = 0.0207$; $p = 0.0017$) in Groups I and II respectively, then rose slightly at 4 and 6 months of age.

ii. In Group I there was a significant decrease between cord anti-AGG2,3 titres and titres at 4 months ($p > 0.05$) and 6 months ($p > 0.05$).

iii. In Group II there was no significant change in anti-AGG2,3 titres between any sampling time interval.

Groups III

i. The anti-AGG2,3 GMT was significantly higher at 4, 6 and 9 months of age than that in earlier Groups I and II ($p = 0.0001$).

ii. There was no significant increase in GMT between any sampling time interval.

Seroconversion rates: Percentage seroconversion in response to A-DTP and W-DTP is shown in

Table 8.16 and Figure 8.10. The percentage of infants with ≥4-fold increases in anti-PT titres by 9 months of age was significantly higher for acellular vs. whole-cell vaccine ($p < 0.05$), but was not significantly different when the former was commenced at birth or at 2 months. PT seroconversion was highest in Group II, slightly lower in Group I and substantially lower in Group III. A lower rate of seroconversion to AGG2,3 was found in recipients of A-DTP than in W-DTP recipients.

i. PT: In Group I the anti-PT seroconversion rate by 9 months of age was 54.2%; 25.8% of infants had a <4-fold rise in titre and 20.0% did not seroconvert. In Group II the seroconversion rate was 75.4%; 17.4% had a <4-fold rise in titre and 7.2% did not seroconvert. In Group III after 3 doses of W-DTP vaccine the rate of PT seroconversion was only 19.0%; 17.2% had a modest rise in titre and the majority (63.8%) did not seroconvert. More than half the seroconverters in Group I achieved an anti-PT level which was more than 10 times higher than their cord blood level.

ii. FHA: In Group I the anti-FHA seroconversion rate by 9 months of age was 23.3%; 34.9% of subjects had a <4-fold rise in titre and 41.8% did not seroconvert. In Group II the rate was 69.2%; 23.1% had a <4-fold increase in titre and 7.7% did not seroconvert. In Group III the rate was only 24.1%; 31.0% had a modest rise in titre and 44.9% did not seroconvert.

iii. AGG2,3: In Group I the anti-AGG2,3 seroconversion rate by 9 months of age was 22.5%; 29.8% had a <4-fold rise in titre and 47.6% had a fall in titre. In Group II, the rate was 22.1%; 47.1% had a <4-fold rise in titre and 30.8% did not seroconvert. In Group III the rate was 67.2%, 16.4% had a <4-fold increase in titre and 16.4% did not seroconvert.

8.7 DISCUSSION

Acellular pertussis vaccines have been shown to be less toxic than conventional whole-cell

vaccines in studies in developed countries (Edwards *et al.*, 1986; Lewis *et al.*, 1986; Blennow *et al.*, 1988) but not in developing countries. Only 1 study of acellular vaccines in Africa has been made to date (Biritwum *et al.*, 1985), in children aged between 3 months and 3 years. It was therefore necessary to address the questions of whether the acellular vaccine would be safe and immunogenic if administered soon after birth and whether it would prove more immunogenic and less toxic than the conventional vaccine when administered according to the routine schedule. The results of this study revealed the following:

i. The acellular vaccine appeared to be safe when administered together with BCG and TOPV before discharge from the neonatal nursery. Acellular-DTP appeared to be safest and most effective when incorporated into routine vaccination schedules.

i. Although specific antibodies to 2 major protective antigens of *B. pertussis*, PT and FHA could be elicited from birth, seroconversion rates and peak response to PT at 6 months of age were lower than those obtained when vaccination commenced at 2 months of age, ie. according to the routine schedule. Antibody production did occur after the neonatal dose of acellular vaccine.

iii. The acellular vaccine appeared to be associated with slightly more minor, but fewer serious, post-vaccination events than the whole-cell vaccine, although statistically significant differences were not demonstrated. No major post-vaccination events occurred when the acellular vaccine was given from 2 months of age.

iv. Two doses of acellular vaccine were found to be sufficient for peak responses; no significant increase in titres occurred after the third or fourth dose.

v. Response to South African whole-cell vaccine was found merely to restore levels of serum IgG to PT and FHA to those found in cord blood.

Recent trials of acellular pertussis vaccines have demonstrated that these confer some level of protection (Aoyama *et al.*, 1985; *Ad hoc* Group for the Study of Pertussis vaccines, 1988) and have shown that increases in serum IgG antibodies to the vaccine antigens viz. PT and FHA are

a feature of the response (Blennow *et al.*, 1986; Sato & Sato, 1985; Izumiya, 1985). Evidence for protection by anti-PT and anti-FHA antibodies have been obtained in mice (Sato *et al.*, 1981; Sato & Sato, 1984; Oda *et al.*, 1984). The acquisition of IgG antibodies to PT and FHA have not however been shown to correlate with clinical immunity or vaccine efficacy in humans, although these are thought to be the major antigens in the production of protective immunity.

The present study was not designed to determine the protective effect of the vaccines used. A recent study by Long *et al.*(1990b) clearly showed that after whole-cell pertussis vaccination, immunity to disease did not necessarily imply protection from infection.

The acellular vaccine induced serum antibody responses to PT and FHA in these African infants comparable to those reported in older Ghanaian and Japanese children (Biritwum *et al.*, 1985; Sato *et al.*, 1984) receiving a similar vaccine preparation. PT and FHA have been shown to be immunogenic constituents of whole-cell DTP (Ashworth *et al.*, 1983). Surprisingly, recipients of this vaccine in the current study produced little or no response to these antigens.

The majority of infants receiving A-DTP, regardless of the number of doses administered, or the level of maternally-acquired antibodies, had a definite immune response to PT and FHA by 6 months of age, ie. after 2 doses of vaccine. Seroconversion rates to PT obtained in Swedish trials of acellular DTP in 18-24 month and 4-6 year old children after 2 doses of vaccine were 59% for W-DTP and 100% for A-DTP (Olin, 1985).

It is however difficult to make direct comparison with antibody levels attained in studies in other countries owing to differences in population groups and size, vaccine manufacture, age groups, antigens, units, methods and reference sera used.

Serospecific protection correlated with serum agglutinin type has been reported in mice (Ashworth *et al.*, 1982). However, no one class or type of pertussis agglutinin antibody has

been demonstrated to be protective in humans and it is unknown whether antibodies to agglutinogens alone are sufficient to confer immunity to pertussis (Pichichero *et al.*, 1987). The response to AGG2,3 in acellular vaccinees in this study was in accordance with reports by Sato *et al.*, (1984), and was, not surprisingly, significantly lower than in whole-cell vaccine recipients since the former contained only negligible quantities of agglutinogens. The modest rise in agglutinogens following A-DTP vaccination in this study may also be ascribed to a cross-reaction induced by FHA.

In view of the reported efficacy of the South African whole-cell pertussis vaccine (Metcalf *et al.*, 1989), it was both surprising and interesting that serum IgG responses to PT and FHA, 2 principle antigens of *B. pertussis* occurred infrequently and that little or no increase in antibody titre was found after 3 doses of the vaccine. A response to AGG2,3 was noted in these vaccinees, although no significant increase in titre occurred between pre-vaccination sera and final sera at 9 months. Several studies have reported serum antibody responses to all 3 antigens following primary vaccination with conventional whole-cell vaccines (Ashworth *et al.*, 1983). Edwards *et al.*, 1989; Sato, 1984; Baraff *et al.*, 1984) which questions the immunogenicity of the South African product.

The protective effect of pertussis vaccination in the young infant has not been adequately resolved. Passively acquired circulating maternal antibodies may provide protection in the first few weeks of life and may also block or modify the serum immune response during this time.

Most previous studies of whole-cell vaccine have demonstrated that although infants less than 2 months old are able to respond to vaccination, the percentage of children responding and the antibody titres achieved are lower than those observed in older children (Brown, 1960,1964; Burstyn *et al.*, 1983; Baraff *et al.*, 1984; Provenzano *et al.*, 1965). Other studies have demonstrated no significant differences in attack rate or subsequent antibody titres when vaccination commenced soon after birth or 2 months later (Schwartz *et al.*, 1985; Abayoni *et al.*,

1982).

One way of improving vaccination coverage rates in developing countries is to simplify the vaccination schedule and hence reduce the number of contacts of the child with the health services. The effect of neonatal vaccination with acellular pertussis vaccines on the serologic response to subsequent doses has not been evaluated previously.

In the present study, acellular vaccination of neonates produced a definite response to PT and FHA, however better serological responses occurred when the vaccine was incorporated into routine schedules commencing at 2 months of age. This muted response may indicate the development of immune tolerance as a result of high levels of pre-existing maternally acquired antibodies. This does not necessarily imply that vaccination is without effect since sensitization may occur.

The incidence of pertussis infection after a primary series of either 2 or 3 doses of high potency whole-cell vaccine was not reported to be significantly different (Muller *et al.*, 1984). An immunogenicity trial of acellular vaccine in Sweden (Blennow *et al.*, 1985) found that antibody responses were independent of schedule, ie. titres after 2 doses given 2 months apart were significantly higher than after 3 doses of plain whole-cell vaccine. Also, no significant difference was noted in PT and FHA titres attained after 2 compared with 3 doses of acellular vaccine, as was the case in the present study. Infants vaccinated at birth received 4 doses of A-DTP whereas infants in the routine vaccination groups received either 3 doses of acellular or whole-cell DTP.

Peak titres and seroconversion rates to PT occurred after 2 doses of A-DTP and did not increase significantly after the third or fourth doses of vaccine. Two doses of A-DTP produced a significantly greater response to PT and FHA than 3 doses of W-DTP. It should be noted that, in the absence of vaccination, antibody should continue to fall from levels seen in cord blood,

hence seroconversion rates might not mean much. The rate of fall is exemplified by children in Group II, not vaccinated at birth (cord and 2-month levels of PT, FHA and AGG2,3 antibodies differed significantly; $p = 0.01$, $p = 0.0001$; $p = 0.002$ respectively).

In addition, poor seroconversion in those with high maternal titres are a reflection of initial antibody levels and do not indicate impaired responsiveness to vaccination. We have therefore emphasized final titres rather than seroconversion in our assessments.

The results of Swedish clinical trials of BIKEN A-DTP indicated significantly fewer mild local and systemic reactions (and a similar side effect profile to a placebo) than obtained with non-adsorbed whole-cell vaccine (Blennow *et al.*, 1985; Olin, 1985). Fever, pain, drowsiness, fretfulness, loss of appetite and local reactions were reported to occur less frequently in 18-24 month old and 4-6 year old recipients of A-DTP compared with recipients of W-DTP (Edwards *et al.*, 1986; Lewis *et al.*, 1986; Pichichero *et al.*, 1987).

Minor local and systemic symptoms were reported more often in A-DTP recipients than in recipients of W-DTP in the present study. Infants receiving a neonatal dose of A-DTP had a lower rate of minor post-vaccination symptoms than those vaccinated from 2 months of age. However more infants (21 vs. 12) were affected in the latter group. The incidence of mild local post-vaccination symptoms was reported to be 6.8/1000 in Ghanain infants vaccinated with BIKEN A-DTP (Biritwum *et al.*, 1985). A similar incidence (5.1/1000 doses) was recorded in the present study. Delayed onset of local reactions, reported to occur 5-7 days following A-DTP (Granström *et al.*, 1987) were not reported to us.

The incidence of local reactions to W-DTP has been reported to vary from 16.7% to 72.2% (Baraff *et al.*, 1984; Cody *et al.*, 1981). Surprisingly no recipient of this vaccine in the present study incurred a local reaction as these are frequently reported after DT alone (Pollock *et al.*, 1984). In accordance with serologic results, this is suggestive of a low potency vaccine. Local

reactions occurred more frequently after the second injection of A-DTP in clinical trials in Sweden (Olin, 1985). On the contrary our findings indicated more local and systemic reactions after the first injection than after subsequent injections when vaccination commenced at 2 months. Infants who received a neonatal dose of A-DTP however incurred more local and systemic reactions after the second injection at 2 months of age. Whole-cell DTP-recipients incurred both major and minor post-vaccination symptoms more frequently after the first injection.

Acellular pertussis vaccines have not been proved to be associated with fewer neurological events than conventional vaccines. Only limited use of the vaccine in children younger than 2 years of age has been made to date (Miller *et al.*, 1991). Febrile reactions are reported to be less frequent following A-DTP than W-DTP. It can therefore be expected that vaccine-associated febrile convulsions will be less common. Major neurologically-associated post-vaccination symptoms noted in this study were convulsions and hypotonic-hyporesponsive episodes. These occurred more frequently in W-DTP vaccinees and did not produce detectable sequelae during the brief period of follow-up. Although no causal relationship to pertussis vaccination was established these symptoms are nonetheless a cause for concern.

Several studies of acellular vs. whole-cell vaccine in older children reported no neurologically-associated post-vaccination events (Edwards *et al.*, 1989; Pichichero *et al.*, 1987; Biritwum *et al.*, 1985). There are no reports of the occurrence of these reactions following A-DTP vaccination of neonates. Three cases of convulsions occurred in this study; 1 in an infant who received A-DTP from birth (1.69/1000 doses) and 2 in W-DTP recipients (8.03/1000). Differences in frequency of these reactions were not demonstrable owing to small numbers in the study. The rate of convulsions in W-DTP recipients in the present study appeared unusually high compared with age-matched studies of the vaccine in the United Kingdom (0.4/1000 doses) (Pollock *et al.*, 1984) and the United States (0.57/1000 doses) (Cody *et al.*, 1981). In our study of measles vaccines among Black children in South Africa (unpublished observations) the

rate of convulsions was 0/136 doses; this post-vaccination event may therefore appear to be limited to vaccination with the South African W-DTP preparation.

The hypotonic-hyporesponsive state appears to be a reaction unique to pertussis vaccine and occurs predominantly in the first year of life. (Cody *et al.*, 1981); its rate of occurrence in acellular vaccinees could not therefore be predicted from Japanese data since this was collected in children. Hypotonic-hyporesponsive episodes were recorded in 2 infants within a week of W-DTP vaccination (4.07/1000 doses) in the present study. In the United States this reaction occurred at a rate of 0.57/1000 doses after W-DTP; no episodes were reported in A-DTP vaccinees (Cody *et al.*, 1981). It is however difficult to compare reaction rates in different studies as the definition of the reaction may differ.

The likelihood of these vaccine-associated neurologic events being due to intercurrent phenomena (unrelated host-problems, factors in infancy) cannot be excluded. Swedish clinical trials of A-DTP indicate a high incidence of random neurologic events (convulsions, cyanosis, nystagmus, temperature $>40^{\circ}C$) (*Ad Hoc* Group for the Study of Pertussis Vaccines, 1988). Because vaccination is usually given at an age when other illnesses are common and when underlying neurologic disorders and defects in development are beginning to show up, there are ample opportunities for chance associations.

The risks of major post-vaccination events cannot be fully quantified in a study of this size and duration, particularly as the study population has a high incidence of infectious and other diseases. The small sample size did not allow statistical evaluation of the rate of occurrence of neurologic symptoms in the various vaccine groups and prevents any conclusion regarding reduced incidence of these symptoms following A-DTP vaccination.

There are no data on the incidence of neurologic events in comparable unvaccinated infants. From the point of view of study design it would have been preferable to include a randomised

control group of children not receiving any pertussis vaccine at all, but this would have been unethical.

Eleven children (3.2%) with pertussis infection were diagnosed during the study period. Only 1 child of the 11 (9.09%) had overt clinical signs of disease and could be diagnosed prospectively on the basis of clinical examination alone. Subclinical pertussis was retrospectively diagnosed in 10 infants, most of whom had not completed primary vaccination. This could mean attenuated disease due to partial immunity (whether vaccine induced or maternally-acquired); or that the disease was milder "*de novo*". All but 1 case occurred in infants who were 4 months of age or younger, which reinforces the need for vaccination very early in infancy.

Overall the malnourished infants responded no less well to pertussis vaccination than did the other vacinees (data presented in Chapter 11).

It is interesting to note the significant antibody levels in maternal sera. Most First World studies have shown a lack of demonstrable pertussis antibodies in women of child-bearing age (Brown, 1960; Burstyn *et al.*, 1983). Since these antibodies are unlikely to be due to the W-DTP currently used in South Africa (given the poor antibody response to PT and FHA shown in this study); it is assumed that the presence of these antibodies are the end result of natural infection and therefore that pertussis is widespread in the African community. This high level of disease may bolster "vaccine" immunity throughout life.

Although the size and design of the present trial prevents a definite conclusion, the results obtained imply that acellular vaccine can be given according to routine schedules with the expectation of unrestricted antibody responses to the principle antigens and that the incidence of post-vaccination events will be no greater than that of conventional W-DTP. These observations are particularly significant in the Third World where pertussis vaccines have to be given early in infancy since morbidity and mortality is highest in this age group.

Further and more extensive efficacy studies are indicated before the use of acellular pertussis vaccines can be recommended for routine primary vaccination of infants in preference to whole-cell preparations in developing countries.

TABLE 8.1 Age-related incidence of intercurrent illnesses.

VACCINE GROUP*	AGE GROUP (months) 0<2	≥2<4	≥4<6	≥6≤9	TOTAL (%)
Group I					
Number of illnesses	15	31	38	75	159
%of total infants with illnesses	3.01	6.22	7.63	15.06	31.93
% of infants with illness in vaccine group	9.43	19.50	23.90	47.17	
% of infants with illness in age group	19.74	27.19	30.40	40.98	
Group II					
Number of illnesses	14	33	45	75	167
%of total infants with illnesses	2.81	6.63	9.04	15.06	33.53
% of infants with illness in vaccine group	8.38	19.76	26.95	44.91	
% of infants with illness in age group	18.42	28.95	36.00	40.98	
Group III					
Number of illnesses	47**	50	42	33**	172
%of total infants with illnesses	9.44	10.04	8.43	6.63	34.54
% of infants with illness in vaccine group	27.33	29.07	24.42	19.19	
% of infants with illness in age group	61.84	43.86	33.60	18.03	
TOTAL NUMBER OF ILLNESSES IN AGE GROUP	76	114	125	183	498
%OF INFANTS WITH ILLNESS IN AGE GROUP	15.26	22.89	25.10	36.76	100.00

* Group I received acellular DTP commencing at birth
Group II received acellular DTP commencing at 2 months of age
Group III received whole-cell DTP commencing at 2 months of age.

** Statistics for table of age group by vaccine group:
chi-square value = 52.016, probability $p < 0.0001$ (sample size = 498).

TABLE 8.2 Intercurrent illnesses in black infants from birth to nine months of age.

INFECTION/ILLNESS	VACCINE GROUP* GROUP I	GROUP II	GROUP III	TOTAL
Upper respiratory tract	57	56	64	177
Skin	31	51	52	134
Lower respiratory tract	12	16	12	40
Diarrhoea	25	16	14	55
Pyrexia of unknown origin	8	9	3	20
Eye	6	5	6	17
Other**	20	14	21	55
Total number of illnesses	159	167	172	498
Total number of infants with illness	66	70	70	206

* Group I Acellular DTP from birth
Group II Acellular DTP from 2 months
Group III Whole-cell DTP from 2 months.

** Viral or bacterial infections and clinical signs unrelated to infections noted above.

TABLE 8.3 Nature of intercurrent illnesses in black infants according to age and vaccine group.

AGE GROUP (mths)	0<2			≥2<4			≥4<6			≥6>9		
VACCINE GROUP	I	II	III	I	II	III	I	II	III	I	II	III
ILLNESSES/INFECTION												
Upper respiratory tract	2	3	20	10	14	18	15	16	14	30	23	12
Skin	2	3	13	10	11	13	6	11	15	13	26	11
Diarrhoea 2	-	1	3	1	6	4	3	5	16	12	2	
Lower respiratory tract	1	-	4	4	3	4	1	7	3	6	6	1
Pyrexia of unknown origin	1	-	1	1	1	2	1	3	0	5	5	0
Eye	1	3	1	2	1	2	3	1	1	0	-	2
Measles -	-	-	-	-	-	-	-	-	2	1	2	
Chicken pox -	-	-	-	1	-	-	-	-	1	1	1	
Viral meningitis	-	-	-	-	-	-	-	1	1	-	1	-
Hepatosplenomegaly	-	1	2	-	1	3	3	3	1	1	-	-
Jaundice 2	2	1	-	-	-	2	-	-	-	-	-	
Ventricular septal defect	-	-	1	-	-	-	-	-	-	-	-	-
CNS-related* 1	2	3	-	-	2	3	-	1	1	-	-	
Anaemia 3	-	-	1	-	-	-	-	-	-	-	-	
Oral herpes -	-	-	-	-	-	-	-	-	-	-	2	
TOTAL ILLNESSES (n)	15	14	47	31	33	50	38	45	42	75	75	33

* Craniotabes; bossing.

TABLE 8.4 Post-vaccination events following administration of acellular pertussis vaccine in very young african infants (doses 1, 2, 3 ± 4 combined).

SYMPTOM	NUMBER OF EVENTS GROUP I (349 doses)	GROUP II (242 doses)	P-VALUE*
Major			
Convulsion	1	0	1.000
Hypotonic-hyporesponsive episode	0	0	-
Systemic			
Excessive crying	10	15	0.047*
Fever	8	10	0.200
Irritability	7	2	0.320
Loss of appetite	2	3	0.404
Local (at injection site)			
Induration	1	2	0.570
Swelling	1	1	1.000
Total events	30	33	
Number of infants with ≥ 1 symptom	12	21	

* Chi-square; $p < 0.05$ denotes a statistically significant difference

Group I A-DTP from birth
Group II A-DTP from 2 months.

TABLE 8.5 Post-vaccination events following administration of acellular or whole-cell pertussis vaccines (doses 1, 2, 3 ± 4 combined).

SYMPTOM	NUMBER OF EVENTS ACELLULAR** (591 doses)	WHOLE-CELL (249 doses)	P-VALUE*
Major			
Convulsion	1	1	1.00
Hypotonic-hyporesponsive episode	0	2	0.09
Systemic			
Excessive crying	25	6	
Fever	18	8	0.9
Irritability	9	1	0.3
Loss of appetite	5	1	0.7
Local (at injection site)			
Induration	3	0	0.6
Swelling	2	0	1.0
Total events	63	19	
Number of infants with ≥ 1 symptom	33	19	

* Chi-square; $p < 0.05$ denotes a statistically significant difference

** Groups I and II.

TABLE 8.6 Subjects with post-vaccination events: Group I.

SUBJECT No	SYMPTOM DOSE 1	DOSE 2	DOSE 3	DOSE 4	TOTAL
1	nil	F,C,I	F,C,I	nil	6
2	nil	F,C,I	C,L	nil	5
3	nil	F,C,I,L,H	nil	nil	5
4	nil	F,C	nil	nil	2
5	nil	nil	C	F	2
6	nil	nil	C,I	nil	2
7	nil	nil	F	S	2
8	nil	C,I	nil	nil	2
9	C	nil	nil	nil	1
10	nil	nil	C	nil	1
11	nil	nil	C	nil	1
12	nil	nil	nil	F	1
TOTAL	1	15	11	3	30

Key C = crying
F = fever
H = hypotonic-hyporesponsive
I = irritability
L = loss of appetite
S = swelling.

TABLE 8.7 Subjects with post-vaccination events: Group II.

SUBJECT No	SYMPTOM DOSE 1	DOSE 2	DOSE 3	TOTAL
1	F,In,C	nil	nil	3
2	F,C,L	nil	nil	3
3	F,C	nil	L	3
4	F,C,	nil	nil	2
5	F,I	nil	nil	2
6	nil	C,L	nil	2
7	C,I	nil	nil	2
8	C	C	nil	2
9	F,C	nil	nil	1
10	C	nil	nil	1
11	C	nil	nil	1
12	C	nil	nil	1
13	C	nil	nil	1
14	nil	C	nil	1
15	nil	nil	I	1
16	F	nil	nil	1
17	F	nil	nil	1
18	C	nil	nil	1
19	S	nil	nil	1
20	nil	nil	F	1
21	F	nil	nil	1
TOTAL	26	4	3	33

Key C = crying
F = fever
I = irritability
In = induration
L = loss of appetite
S = swelling.

TABLE 8.8 Dose-related incidence of post-vaccination events in recipients of acellular pertussis vaccine commenced at birth (Group I).

	INCIDENCE/1000 DOSES (n)			
	DOSE 1 (n=107)	DOSE 2 (n=92)	DOSE 3 (n=83)	DOSE 4 (n=67)
Major				
Convulsions	0	0	0	0
Hypotonic-hyporesponsive episodes	0	10.9(1)	0	0
Systemic				
Excessive crying	9.35(1)	54.5(5)	72.3(6)	0
Fever	0	43.5(4)	24.1(2)	14.9(1)
Irritability	0	32.6(3)	24.1(2)	14.9(1)
Loss of appetite	0	10.9(1)	12.0(1)	0
Local (at injection site)				
Induration	0	10.9(1)	0	0
Swelling	0	0	0	14.9(1)
TOTAL	1	15	11	3

TABLE 8.9 Dose-related incidence of post-vaccination events in recipients of acellular pertussis vaccine commenced at 2 months (Group II).

	INCIDENCE/1000 DOSES (n)		
	DOSE 1 (n=107)	DOSE 2 (n=92)	DOSE 3 (n=83)
Major			
Convulsions	0	0	0
Hypotonic-hyporesponsive episodes	0	0	0
Systemic			
Excessive crying	137.9(12)	37.0(3)	0
Fever	103.4(9)	0	1.35(1)
Irritability	11.5(1)	0	1.35(1)
Loss of appetite	11.5(1)	12.3(1)	1.35(1)
Local (at injection site)			
Induration	23.0(2)	0	0
Swelling	11.5(1)	0	0
TOTAL	26	4	3

TABLE 8.10 Dose-related incidence of post-vaccination events in recipients of acellular and whole-cell pertussis vaccine.

VACCINE GROUP (Dose)	POST-VACCINATION EVENTS INCIDENCE PER 1000 DOSES (n) MAJOR	LOCAL&SYSTEMIC	ALL
Whole-cell*			
Dose 1 (91)	21.98 (2)	109.89 (10)	131.9 (12)
Dose 2 (83)	0	48.19 (4)	48.2 (4)
Dose 3 (75)	13.33 (1)	26.67 (2)	40.0 (3)
Acellular from birth			
Dose 1 (107)	0	93.46 (1)	9.3 (1)
Dose 2 (92)	10.87 (1)	163.04 (15)	173.9 (16)
Dose 3 (83)	0	120.48 (10)	120.5 (10)
Dose 4 (67)	0	44.78 (3)	44.8 (3)
Acellular from 2 months*			
Dose 1 (187)	0	298.85 (26)	298.9 (26)
Dose 2 (81)	0	49.38 (4)	49.4 (4)
Dose 3 (74)	0	40.54 (3)	40.5 (3)
Acellular (combined)			
Dose 1 (194)	0	139.18 (27)	45.7 (27)
Dose 2 (173)	5.78 (1)	109.82 (19)	33.8 (20)
Dose 3 (157)	0	82.80 (13)	22.0 (13)

* No vaccination at birth.

TABLE 8.11 Statistical analysis* of geometric mean pertussis IgG antibody titres in acellular and whole-cell vaccines (Group I vs Group II vs Group III).

SAMPLING TIME	ANTIBODY PT	FHA	AGG2,3
Birth	F = 0.07 P = 0.93	F = 0.21 P = 0.81	F = 2.52 P = 0.083
4 months	F = 11.90 P = 0.0001[a]	F = 2.52 P = 0.05[d]	F = 4.62 P = 0.011
6 months	F = 16.76 P = 0.0001[b]	F = 1.65 P = 0.19	F = 13.04 P = 0.0001
9 months	F = 16.14 P = 0.0001[c]	F = 4.89 P = 0.009[e]	F = 14.82 P = 0.0001

* Duncans' Multiple Range Test ($p \leq 0.05$ is deemed to be a statistically significant difference).

** Group I Acellular DTP from birth
Group II Acellular DTP from 2 months
Group III Whole-cell DTP from 2 months

a,b,c Group III GMT was significantly lower than Groups I and II
d Group II GMT was significantly lower than Group I
e Group III GMT was significantly lower than Group II.

TABLE 8.12 Geometric mean titres (GMTs) of pertussis IgG-anti-PT antibodies to acellular and whole-cell pertussis vaccines.

BLOOD SAMPLING	ELISA U/ml GROUP I	GROUP II	GROUP III
Maternal serum	42.7 ± 5.7(93)° [1.2-279.5]	52.4 ± 15.4(76) [1.7-1036.9]	41.6 ± 6.5(55) [2.3-211.4]
Cord serum	44.3 ± 7.4(94)+ [0.4-631.5]	48.8 ± 11.4(75) [2.2-608.6]	48.2 ± 9.8(56) [1.4-466.6]
Two months	37.9 ± 5.2(69) [0.9-254.2]	18.5 ± 3.4[a]*(64)+ [0.9-144.3]	31.7 ± 10.3+(49) [1.0-484.0]
Four months	102.5 ± 14.9[b]*(73) [1.1-818.0]	78.5 ± 9.7[c]*(74) [4.6-497.0]	20.5 ± 6.2(54) [0.9-310.9]
Six months	138.0 ± 23.2(60) [2.0-903.0]	183.4 ± 23.0[d]*(68) [12.0-970.9]	23.4 ± 6.9(57) [1.0-290.0]
Nine months	117.0 ± 19.4(52) [1.5-879.0]	136.4 ± 15.5[e]*(57) [10.1-710.4]	18.4 ± 4.2(45) [1.2-186.2]

° GMT ± SEM (n) [range]

* Indicates significant difference ($p < 0.05$) from previous sampling time (unpaired 't' test)

\+ pre-vaccine sera

a: $p = 0.01$
b: $p = 0.0001$
c: $p = 0.0001$
d: $p = 0.0001$
e: $p = 0.002$

Group I A-DTP from birth
Group II A-DTP from 2 months of age
Group III W-DTP from 2 months of age

TABLE 8.13 Geometric mean titres (GMTs) of pertussis IgG-anti-FHA antibodies after acellular and whole-cell pertussis vaccination.

BLOOD SAMPLING	ELISA U/ml GROUP I	GROUP II	GROUP III
Maternal serum	82.9 ± 10.2°(93) [5.5-618.0]	80.4 ± 10.6(74) [12.7-590.3]	72.2 ± 12.1(56) [12.3-591.0]
Cord serum	93.0 ± 17.4(94) [6.2-1376.5]	86.1 ± 10.2(73) [4.9-461.9]	78.5 ± 15.1(55) [5.3-595.4]
Two months	71.9 ± 28.9(68) [1.5-1986.3]	30.1 ± 3.8[a]*(57) [3.9-131.2]	82.8 ± 46.0(50) [1.6-135.2]
Four months	92.6 ± 15.4(73) [2.8-683.5]	49.1 ± 4.5[b]*(66) [3.6-159.4]	68.3 ± 20.0(54) [0.9-325.9]
Six months	145.5 ± 52.8(59) [5.7-3120.0]	107.2 ± 12.9[c]*(59) [14.2-607.9]	62.5 ± 15.0(60) [0.9-265.6]
Nine months	84.5 ± 11.2(50) [11.0-485.9]	486.0 ± 14.3*(49) [9.7-486.0]	60.8 ± 12.5(50) [0.9-183.6]

° GMT ± SEM (n) [range]

* Indicates significant difference ($p < 0.05$) from previous sampling time (unpaired 't' test)

+ pre-vaccine sera

a: $p = 0.0001$
b: $p = 0.0003$
c: $p = 0.0001$

Group I A-DTP from birth
Group II A-DTP from 2 months of age
Group III W-DTP from 2 months of age

TABLE 8.14 Pertussis IgG-anti-AGG2,3 antibody production after acellular and whole-cell pertussis vaccination.

BLOOD SAMPLING	ELISA U/ml GROUP I	GROUP II	GROUP III
Maternal serum	173.8 ± 28.8°(91) [0.6-1666.3]	189.8 ± 33.3(74) [0.6-1552.4]	404.9 ±203.4(55) [15.7-11228.8]
Cord serum	143.7 ± 26.7(92) [0.6-1667.8]	143.7 ± 26.9(74) [0.6-1657.4]	522.5 ±290.5(54) [0.6-13709.0]
Two months	69.1 ± 12.2[a]*(66) [0.6-488.5]	54.3 ± 9.0[b]*(59) [0.6-348.1]	205.6 ± 94.9(48) [6.1-4109.4]
Four months	105.8 ± 23.7(71) [0.6-1280.2]	54.3 ± 7.9(66) [0.6-315.7]	347.5 ±143.7(50) [0.6-6344.9]
Six months	129.2 ± 27.2(58) [0.6-1064.5]	66.45± 9.9(62) [0.6-390.5]	486.9 ±110.8(54) [069-4310.3]
Nine months	138.3 ± 29.6(49) [0.6-920.0]	94.0 ± 22.3(40) [0.6-1030.4]	714.3 ±160.2(44) [0.6-5785.8]

° GMT ± SEM (n) [range]

* Indicates significant difference ($p < 0.05$) from previous sampling time (unpaired 't' test)

+ pre-vaccine sera a: $p = 0.02$
b: $p = 0.002$

Group I A-DTP from birth
Group II A-DTP from 2 months of age
Group III W-DTP from 2 months of age

TABLE 8.15 Statistical analysis of change in pertussis IgG antibody geometric mean titres (GMTs) between pre-* and post-vaccination sera taken at 4, 6 and 9 months of age (paired t-test).

SAMPLING TIME	P-VALUE		
	Anti-PT	Anti-FHA	Anti-AGG2,3
4 months			
Group I	0.0001(↑)	**	**
Group II	**	0.005(↓)	0.0001(↓)
Group III	0.0001(↓)	**	**
6 months			
Group I	0.0005(↑)	**	**
Group II	0.0001(↑)	**	0.03(↓)
Group III	**	**	**
9 months			
Group I	0.0004(↑)	**	**
Group II	0.001(↑)	**	**
Group III	0.005(↓)	**	**

* Pre-vaccination sera: Group I: cord blood; Group II, III 2-month samples.
** Denotes no significant difference from pre-vaccination titre.
↑ ↓ Denotes increase or decrease from pre-vaccination level.

TABLE 8.16 Seroconversion* in response to pertussis vaccination.

	PERTUSSIS IgG ANTIBODY		
	ANTI-FHA	ANTI-PT	ANTI-AGG2,3
Group I			
% seroconversion	23.3	54.2	22.6
% with slight response	34.9	25.8	29.8
% with no response	41.8	20.0	47.6
Group II			
% seroconversion	69.2	75.4	22.1
% with slight response	23.2	17.4	47.1
% with no response	7.7	7.2	30.8
Group III			
% seroconversion	24.1	19.0	67.2
% with slight response	31.0	17.2	16.4
% with no response	44.9	63.8	16.4

Group I Acellular DTP from birth
Group II Acellular DTP from 2 months
Group III Whole-cell DTP from 2 months

* ≥ 4-fold rise in titre from pre-vaccination titres; a 'slight response' was defined as a < 4-fold rise in titre from pre-vaccination titre.

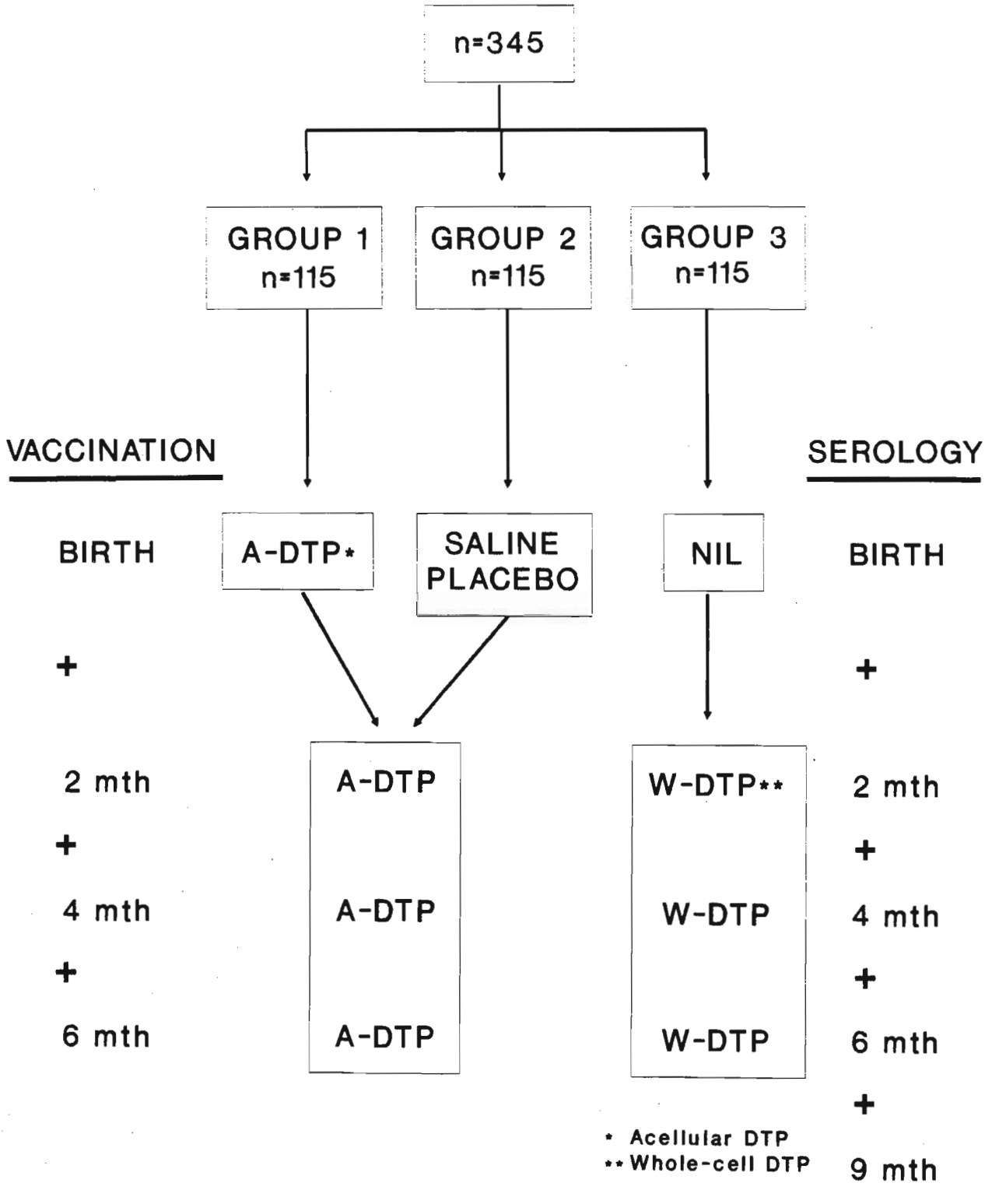


Figure 8.1 Study design.

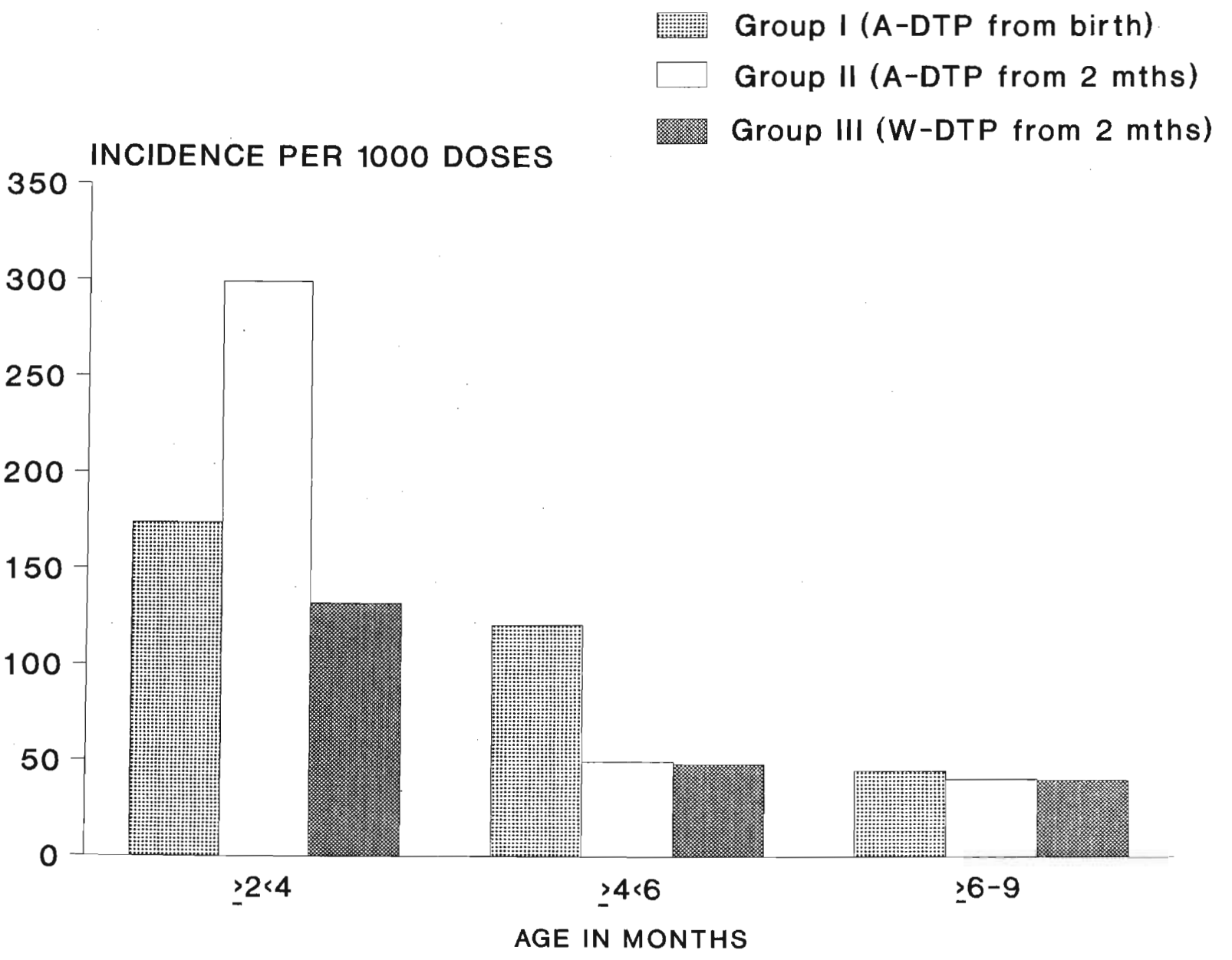


Figure 8.2 Age-related incidence of post-vaccination events following acellular and whole-cell pertussis vaccination.

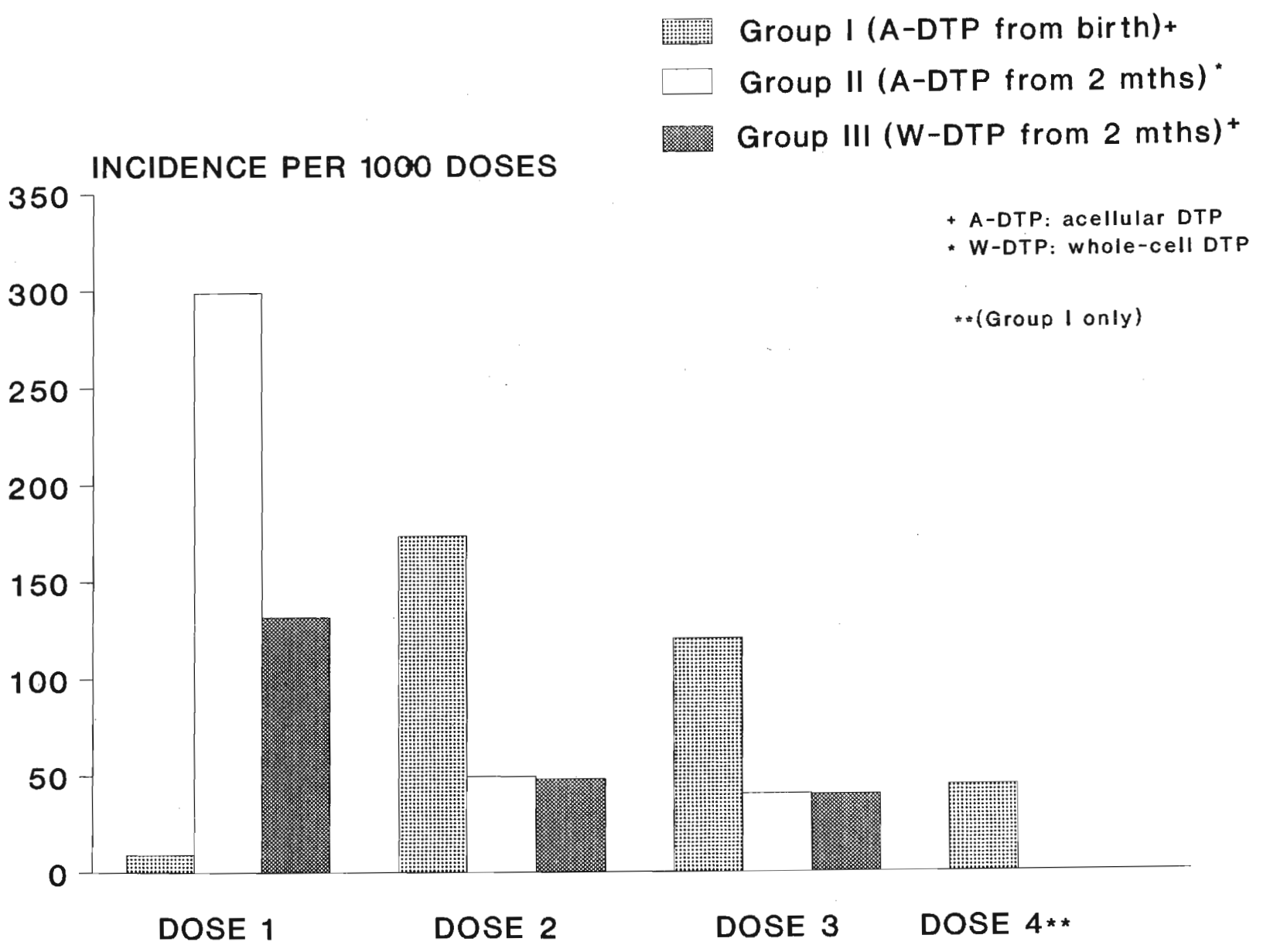
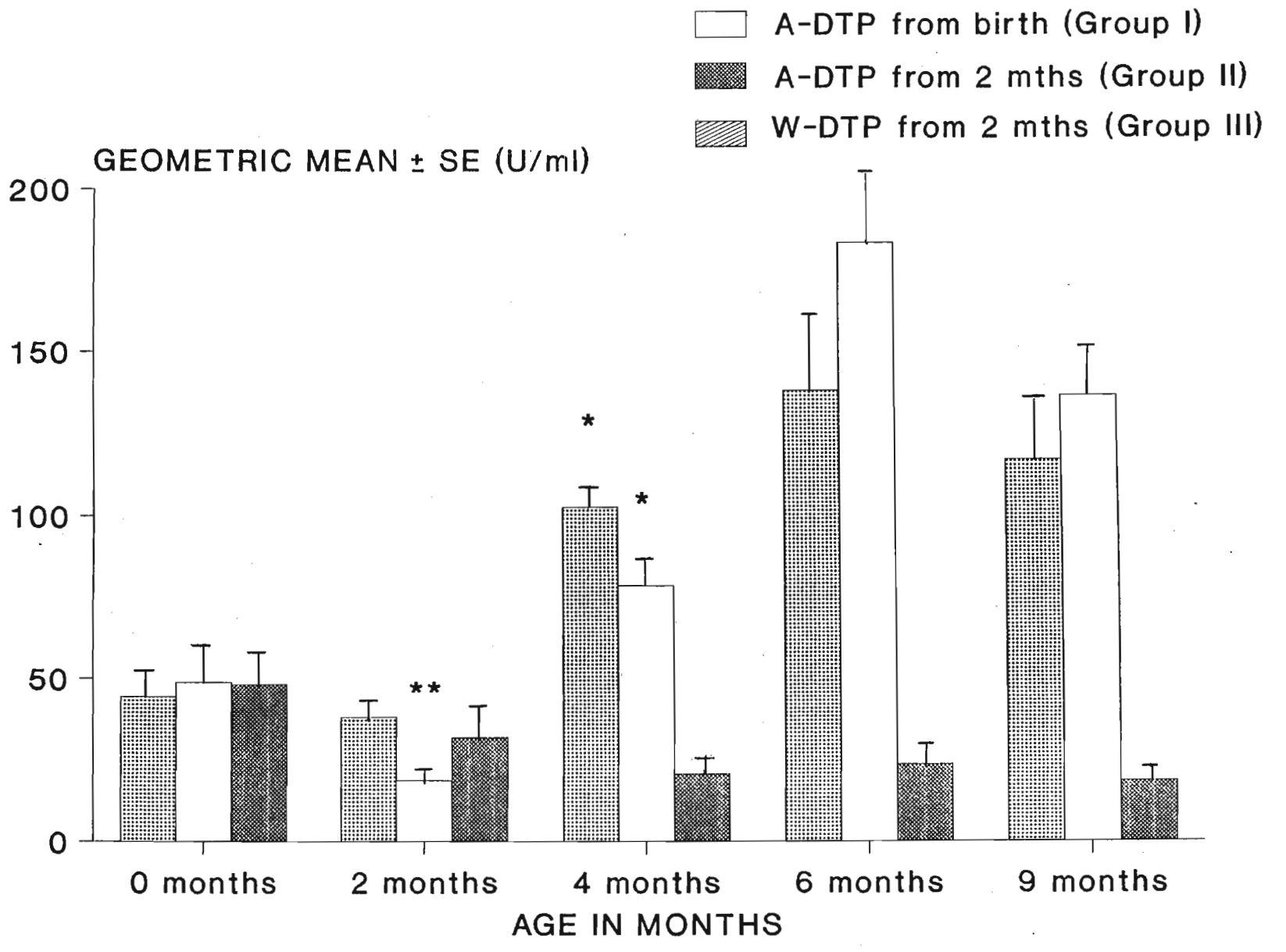
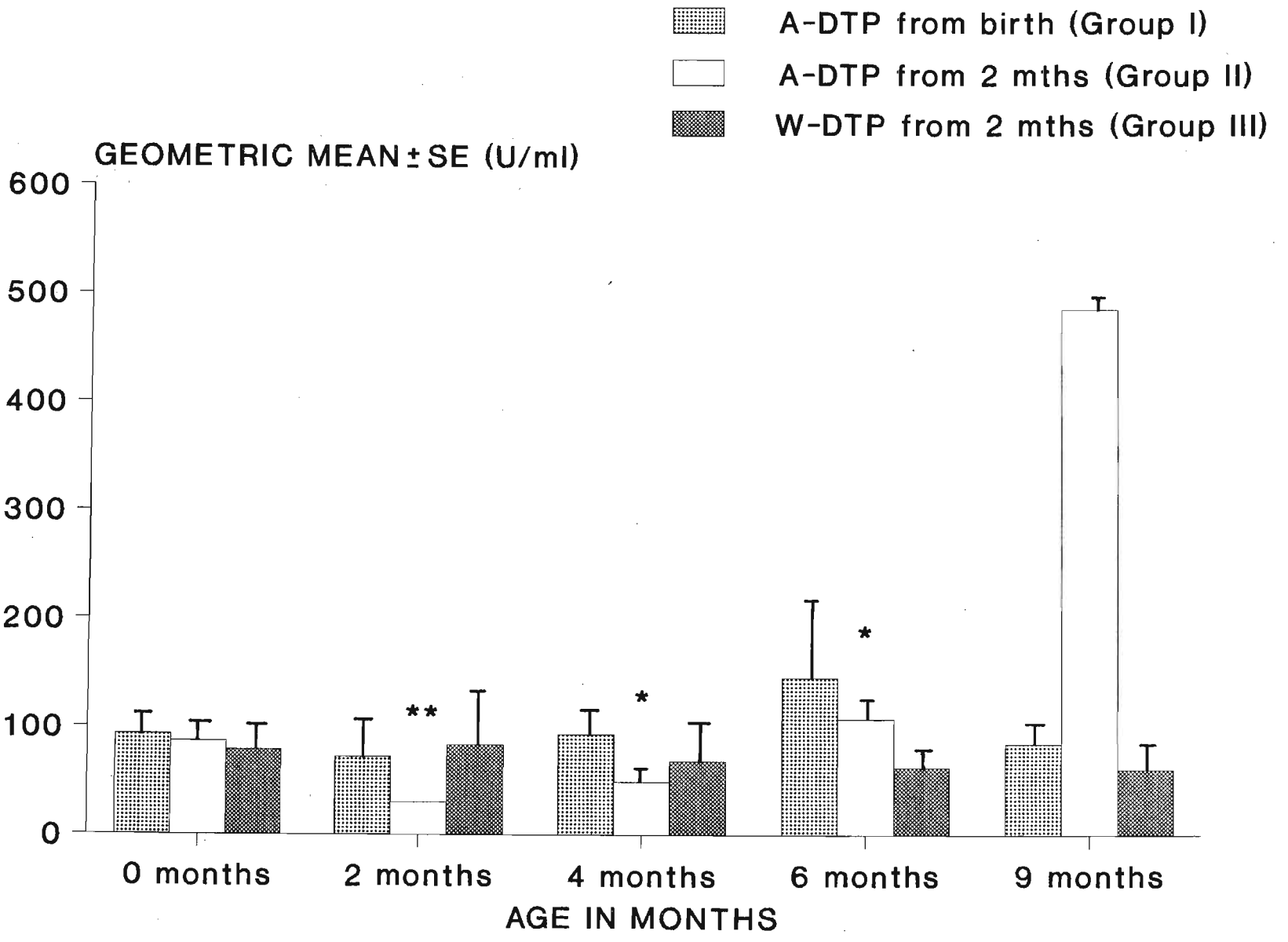


Figure 8.3 Incidence of post-vaccination events following acellular and whole-cell pertussis vaccines (per 1000 doses).



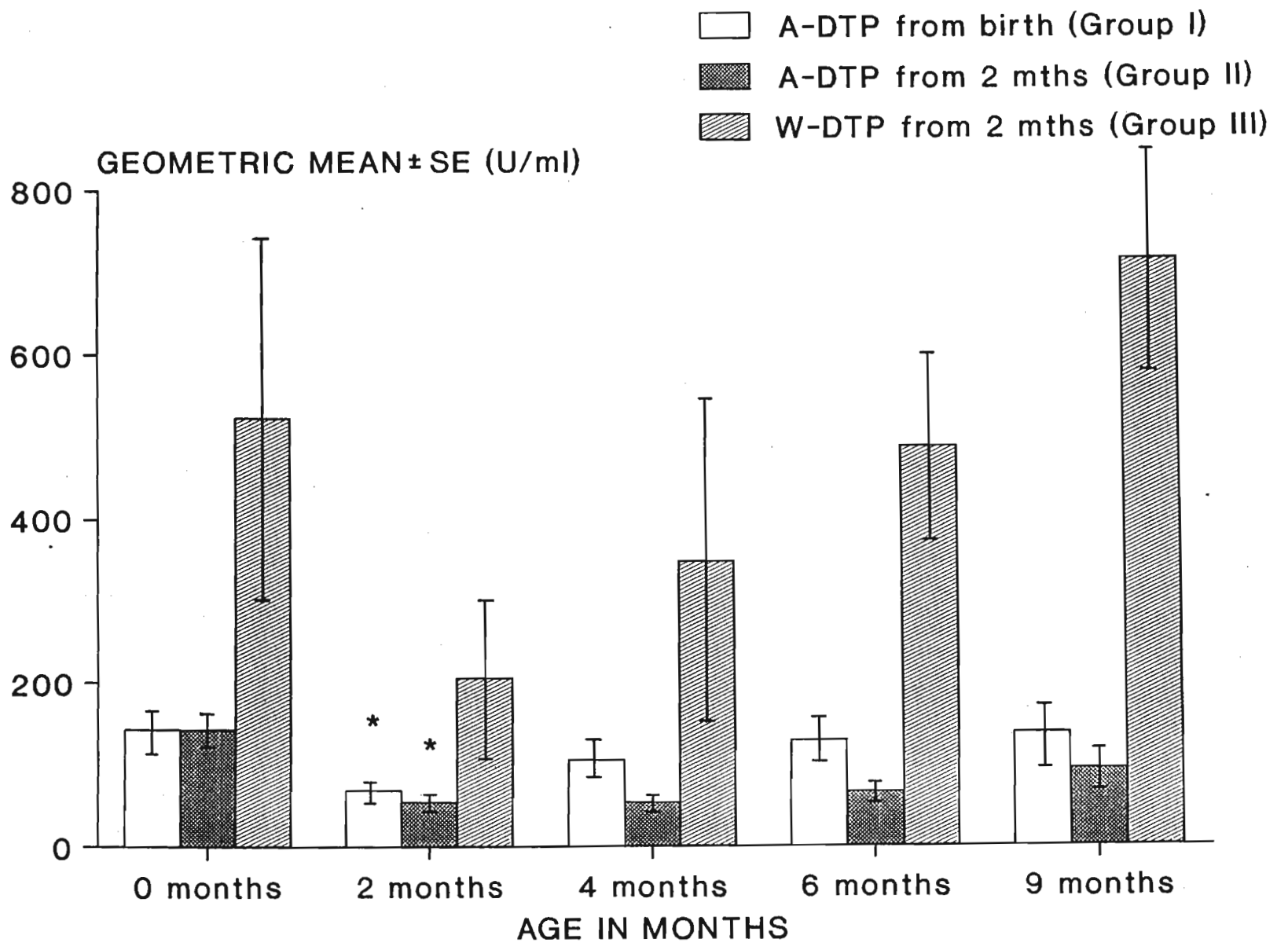
• Indicates significant increase (•) or decrease (••) from previous sampling time ($p < 0.05$) (unpaired 't' test)

Figure 8.4 Pertussis IgG-anti-PT antibody production following acellular or whole-cell pertussis vaccination.



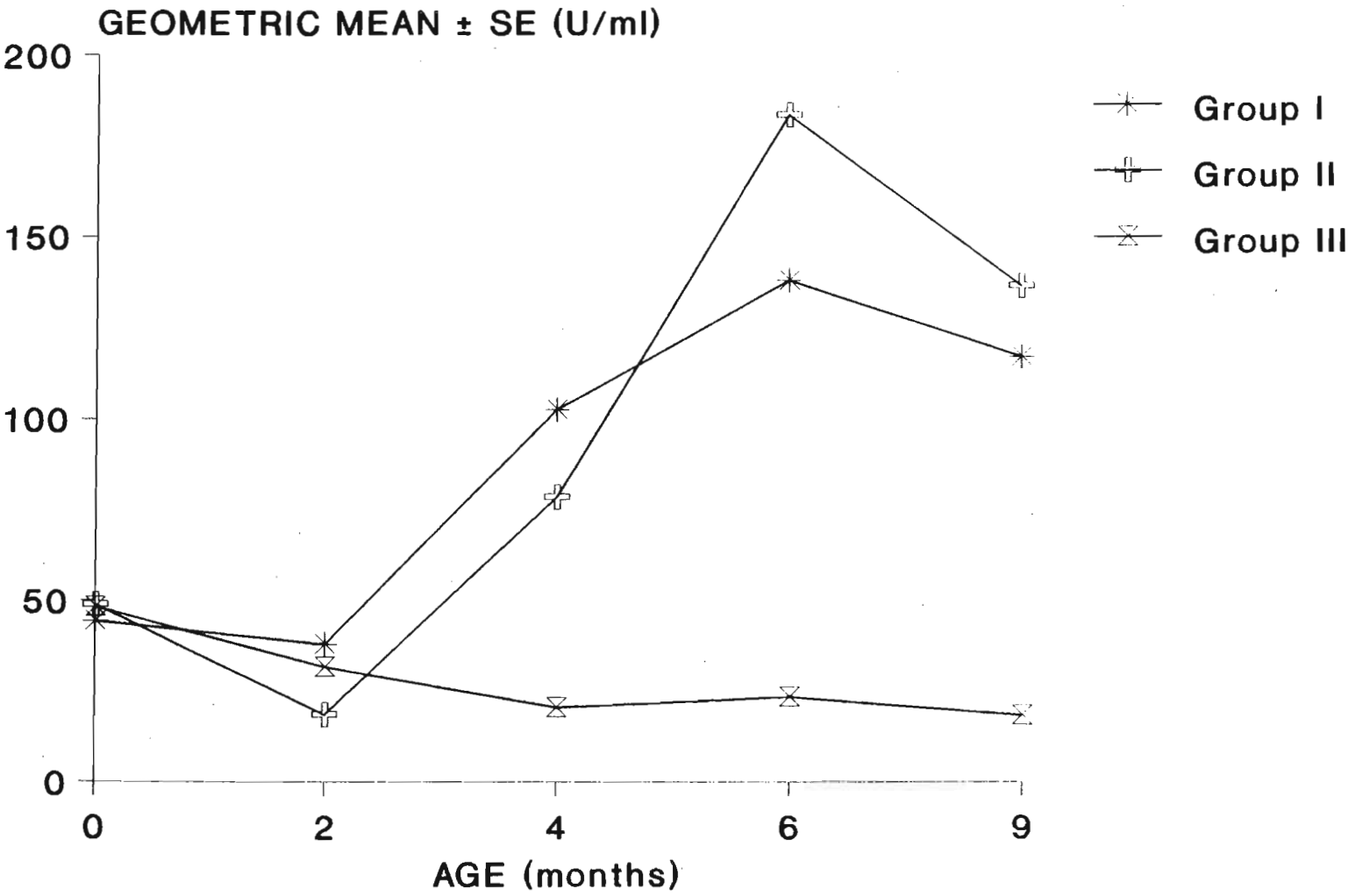
* Indicates significant increase (*) or decrease(**) from previous sampling time ($p < 0.05$) (unpaired 't' test)

Figure 8.5 Pertussis IgG-anti-FHA antibody production following acellular or whole-cell pertussis vaccination.



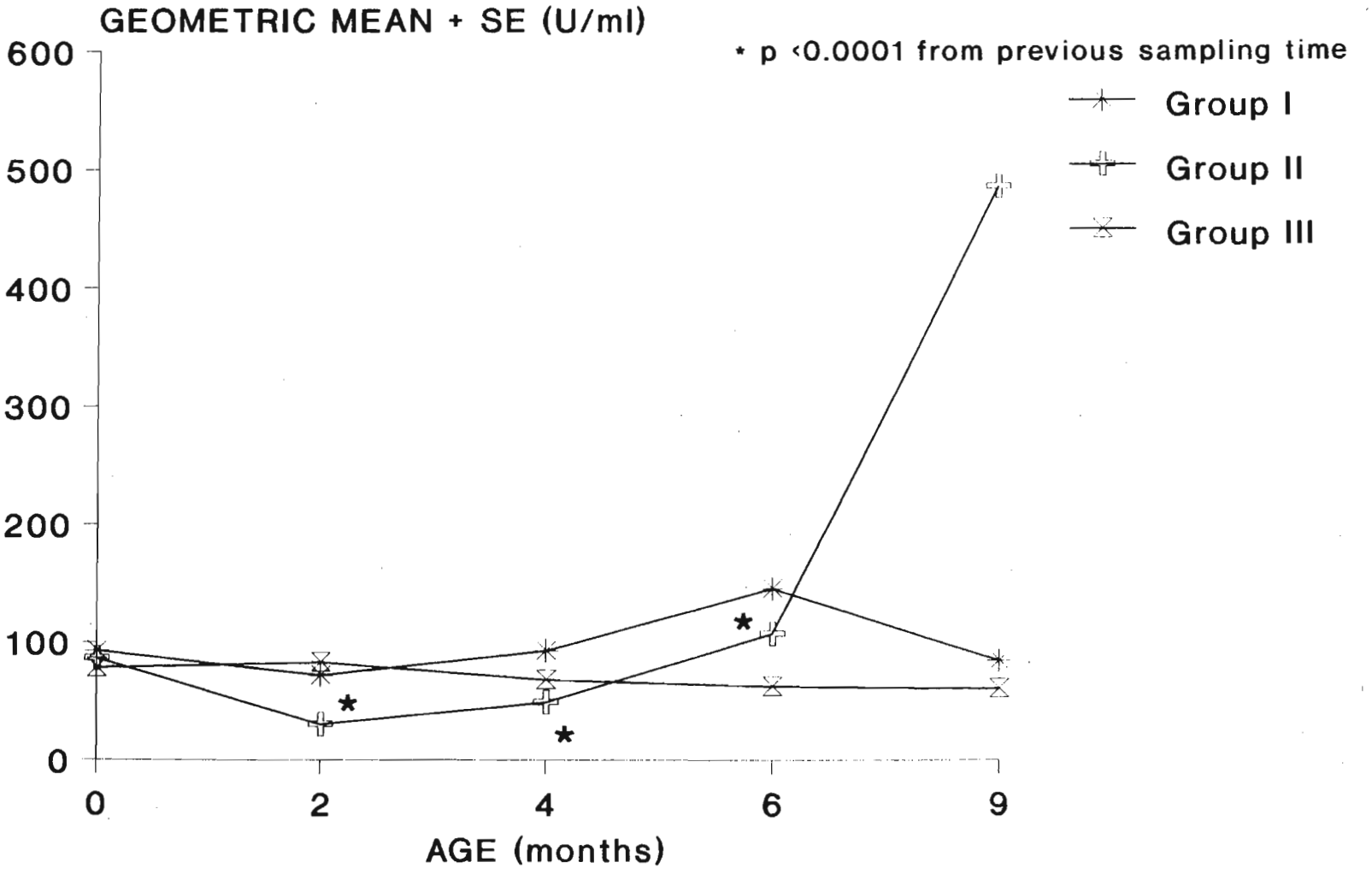
* Indicates significant decrease from previous sampling time ($p < 0.05$) (unpaired 't' test)

Figure 8.6 Pertussis IgG-anti-AGG2,3 antibody production following acellular or whole-cell pertussis vaccination.



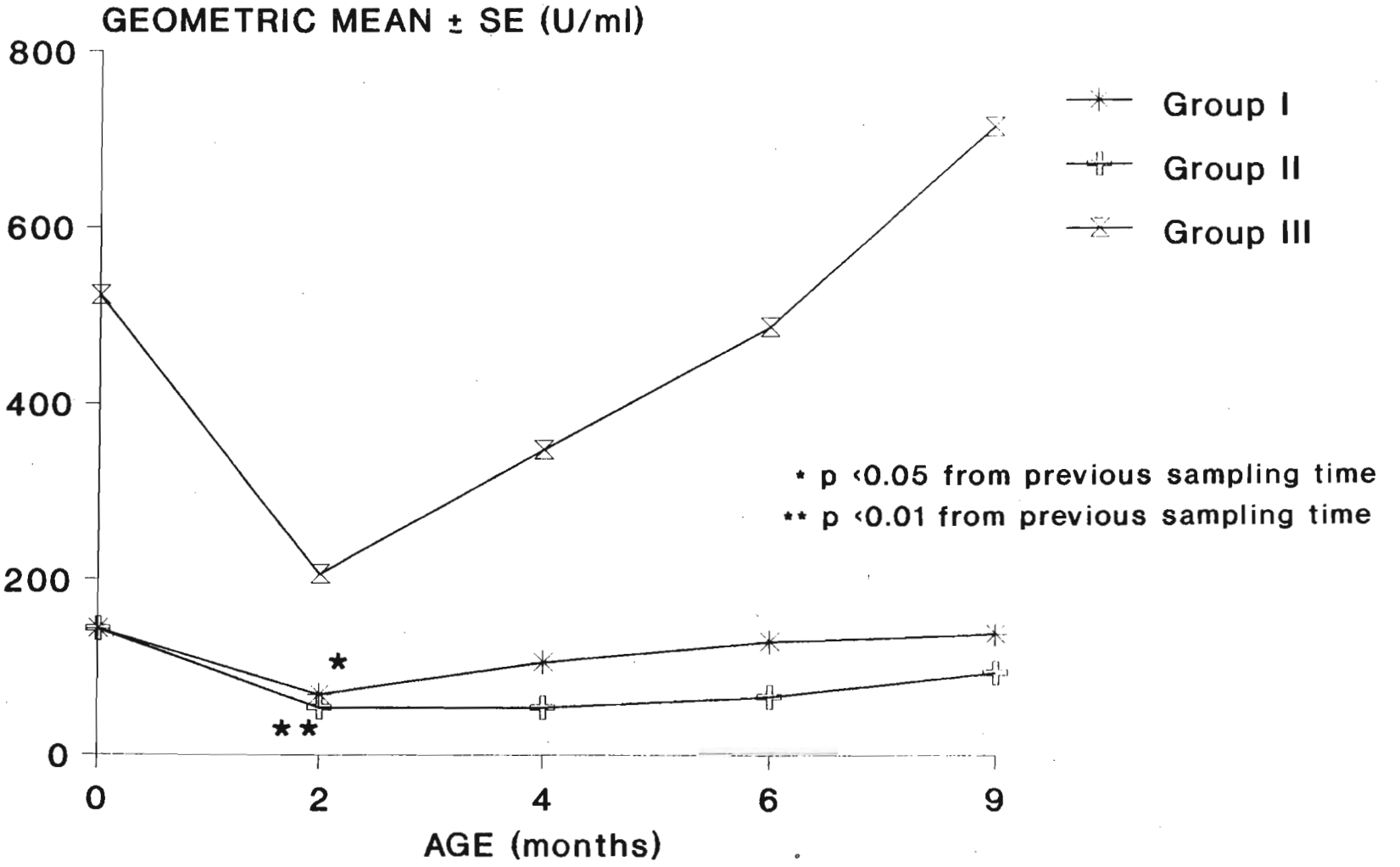
Group I: A-DTP from birth; Group II: A-DTP from 2 mth; Group III: W-DTP from 2 mth.

Figure 8.7 IgG-anti-PT response following acellular and whole-cell pertussis vaccination of African infants.



Group I: A-DTP from birth; Group II: A-DTP from 2 mth; Group III: W-DTP from 2 mth.

Figure 8.8 IgG-anti-FHA response following acellular and whole-cell pertussis vaccination of African infants.



Group I: A-DTP from birth; Group II: A-DTP from 2 mth; Group III: W-DTP from 2 mth.

Figure 8.9 IgG-anti-AGG2,3 responses following acellular and whole-cell pertussis vaccination in African children.

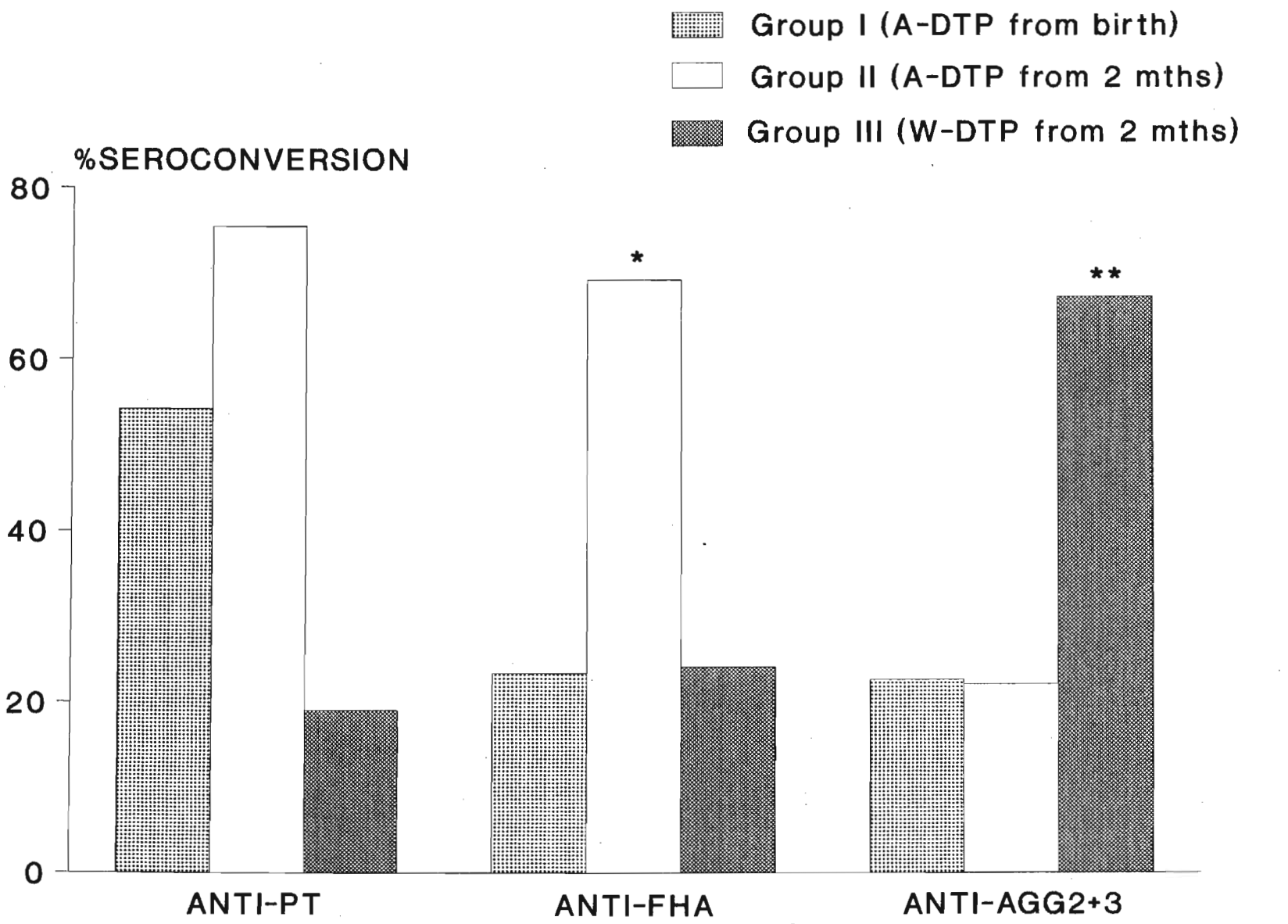


Figure 8.10 Seroconversion*** following acellular or whole-cell perttussis vaccination of African infants from birth to 9 months of age.

CHAPTER 9

SUBCLINICAL PERTUSSIS IN INCOMPLETELY VACCINATED AND UNVACCINATED INFANTS

9.1 OBJECTIVE

To analyse some factors associated with subclinical pertussis infection in very young vaccinated and partially vaccinated infants.

9.2 SUMMARY

The current study identified the role of different factors in subclinical pertussis viz age and gender, nutritional status, vaccination status, household contacts, maternally-acquired antibody levels and humoral immunity. Whilst analysing the results obtained in a phase II study of acellular and whole-cell pertussis vaccines, (Chapter 8), subclinical pertussis was retrospectively diagnosed in 10 infants on the basis of serologic evidence. IgG and IgA to filamentous haemagglutinin (FHA), pertussis toxin (PT) and agglutinogens 2,3 (AGG2,3) were assayed by ELISA in sera obtained at birth, 2, 4, 6 and 9 months of age. All 10 infants had ≥ 4-fold rise in at least 2 different pertussis IgG antibodies. Nine of the 10 infants had ≥ 4-fold increases in all 3 IgG antibodies measured. One infant had ≥ 4-fold increases in IgG antibodies to FHA and AGG2,3 but not PT. Seven infants had a raised IgA antibodies to PT and FHA and 4 infants had raised IgA antibodies to AGG2,3. Subclinical infection provoked antibody production to multiple antigens to differing degrees.

Subclinical infection was detected in both unvaccinated infants (4) and in infants who had been vaccinated with either acellular (4) or whole-cell vaccines (2) commencing at 2 months of age. Subjects were 8 months of age or younger and only 1 had completed primary vaccination. Other infections of infancy were commonly detected; 4 infants had upper respiratory disease about the time of subclinical pertussis. None had a household member with symptomatic pertussis.

Likelihood of subclinical infection was related to significantly lower levels of maternally-acquired pertussis IgG-AGG2,3 antibodies but not associated with infants' nutritional status.

Subclinical pertussis is described in very young babies at an age when the disease is most severe, and therefore has implications for infant morbidity and mortality; it is also relevant to disease surveillance and vaccine-efficacy studies.

9.3 INTRODUCTION

Subclinical pertussis is important for a number of reasons: It may cause silent spread of infection in the community and amongst household contacts; it may be a major cause of unrecognized infant morbidity and mortality; it distorts surveillance data and influences vaccine-efficacy studies (although the latter is conventionally defined in terms of protection against clinical disease). Furthermore, this muted expression of disease consolidates our information on the clinical spectrum of pertussis and raises interesting possibilities with respect to incidence and prevalence of the disease.

There is persuasive evidence that pertussis infection without disease (subclinical whooping cough) does in fact occur. Recent studies have shown subclinical disease in vaccinated infants, health personnel and medical employees, and 'inner-city children' (Long *et al.*, 1990a; Halperin *et al.*, 1989; Linnemann *et al.*, 1975). One-third to two-thirds of infection in vaccinated contacts of pertussis is subclinical (Mertsola *et al.*, 1983; Steketee *et al.*, 1988; Fisher *et al.*, 1989; Long *et al.*, 1990b).

Most evidence of subclinical infection has been established among vaccinated persons (older children and adults), given conventional whole-cell pertussis vaccines in richer industrialized countries.

During analysis of the data obtained in a phase II study of the immunogenicity and safety of acellular and whole-cell pertussis vaccines (Chapter 8) subclinical pertussis was detected among 10 of 342 infants below the age of 9 months on the basis of serologic evidence which

was retrospectively analysed. Various factors in these babies which might account for development of infection without disease were analysed. Special features of the study which I wish to emphasize are:

- pertussis remained subclinical in unvaccinated babies.
- subclinical infection followed incomplete primary vaccination with either acellular or whole-cell pertussis vaccines, and.
- the problem is described in very young African babies at an age when clinical disease is most severe, and therefore has implications for infant morbidity and mortality.

9.4 METHODS

Details of patients and methods are provided in Chapters 4 and 5, hence a very brief account is given here in this section so that this chapter may be read as a whole.

9.4.1 STUDY DESIGN

The data analysed in this paper were collected as part of a large blinded uncontrolled study of the antibody responses and post-vaccination events following primary acellular and whole-cell pertussis vaccination. From March to May 1988, 345 healthy full-term newborn infants from Kwa Mashu, a peri-urban suburb of Durban, inhabited exclusively by blacks, were enrolled in the study after parental informed consent was obtained.

On entry, infants were assigned in sequence of birth to 1 of 3 groups of 115 children each, for receipt of either whole-cell (1 group) or acellular diphtheria-tetanus-pertussis (DTP) (2 groups) at 2, 4 and 6 months according to routine vaccination schedules. In addition, at birth, one group of acellular vaccine recipients received a neonatal dose of this vaccine and the second group, a saline placebo injection. All infants received TOPV and BCG at birth. Infants were asked to return to the clinic at 2, 4, 6 and 9 months of age.

Blood sampling was obtained at birth (mothers' blood and cord blood) and immediately before vaccination at every clinic visit. Sera thus obtained were coded and frozen at -20°C until antibody assays could be performed.

Three vaccine-unrelated deaths occurred in infants younger than 2 months of age. All had been assigned to the whole-cell vaccine group, and therefore had received only BCG and TOPV at birth. Of the 342 remaining subjects, 75% returned at 2 months of age, 68% at 4 months, 58% at 6 months and 51% at 9 months of age.

9.4.2 CLINICAL ASSESSMENT

All infants were monitored for inter-current illnesses and vaccine-associated symptoms up to the age of 9 months through home-visits by a community health nurse with access to tertiary health care. Parents recorded post-vaccination events on a specially designed questionnaire (Appendix 1). These were reviewed at each clinic visit by study personnel.

In addition, all infants underwent assessment of nutritional status and physical examination by a pediatrician at clinic visits at 2, 4, 6 and 9 months. Mothers were questioned about the occurrence and nature of any clinical problems and household contacts of pertussis at this time. Data were recorded immediately on subjects chart.

9.4.3 SEROLOGIC ASSAYS

Serum IgG and IgA antibodies to FHA, PT and AGG2,3 were assayed by micro-ELISA at the Centre for Applied Microbiology and Research (CAMR), Public Health Laboratory Services, Porton UK by the author. The ELISA procedure used was essentially as described by Rutter *et al.* (1988).

Antigens: FHA and PT for use as antigens in the assays were purchased from the Research

Foundation for Microbial Diseases of Osaka University Japan. Co-purified AGG2,3 was provided by Dr. A. Robinson, Porton UK.

Reference serum: Japanese Reference Pertussis Anti-Serum (Human) was a gift from the Research Foundation for Microbial Diseases of Osaka University, Japan. It was supplied from a single lot and contained 250 ELISA units of PT-IgG antibody and 400 ELISA units of FHA-IgG antibody to pertussis per ml. The reference serum was assigned a value of 400 ELISA units/ml of IgG anti-AGG2,3. CAMR IgA Reference Standard was used in IgA assays. The unitage of the test serum relative to the reference serum was calculated by means of parallel-line assays. Results were expressed in ELISA units/ml.

All sera from 1 individual were tested in the same assays on the same day. In some cases the quantity of serum was not sufficient to carry out all the tests required and therefore the number of samples giving rise to the data shown in the tables are not uniform.

9.4.4 DEFINITION OF SUBCLINICAL PERTUSSIS

Subclinical pertussis was defined as serological evidence of infection in the absence of the typical clinical picture of pertussis.

Pertussis infection was defined as 'symptomatic' on observation (or recent history) of prolonged paroxysmal cough, whoop, or cough with associated vomiting, cyanosis, apnoea, sub-conjunctival haemorrhage, epistaxis or periorbital oedema. In the absence of these well-recognised clinical signs, serologic evidence of 'probable pertussis' infection was defined as a significant, ie. ≥ 4-fold increase in value of IgG antibodies to AGG2,3, and to either or both PT and FHA between 2 consecutive serum samples taken 8-12 weeks apart. 'Definite' serologic evidence of pertussis infection was defined as the presence of IgA antibodies to FHA and/or PT, in addition to a significant ≥ 4-fold increase in value of IgG to AGG2,3 and to either or both PT and FHA.

In fact we found at the completion of the study that all 10 had "definite" serologic evidence of

pertussis.

9.4.5 ASSESSMENT OF CHARACTERISTICS OF INFANTS WITH SUBCLINICAL INFECTION

The role of various factors which may have contributed to asymptomatic infection were analysed, viz -

i. household contacts.
ii. type of antibody responses (clinical vs subclinical; vaccinated vs subclinical).
iii. maternally acquired antibody levels.
iv. vaccination status (number of vaccine doses received).
v. age and gender.
vi. nutritional status.

Age and vaccine-matched infants with neither 'definite' nor 'probable' evidence of pertussis infection served as a control group.

9.4.6 STATISTICAL METHODS

Calculations of results and analysis of ELISA antibody values were performed on logarithmically transformed data. Geometric mean antibody values and standard error were calculated. Unpaired and paired t-tests were used respectively to compare mean cord and maternal antibody values between clinical groups and change in an individuals values over time. Sample size was too small to allow statistical evaluation of differences in pre-infection, peak and final antibody titres in infected infants compared with uninfected cohorts. Statistical significance at a 95% confidence level was $p < 0.05$ (2-tailed).

9.5 RESULTS

9.5.1 SUBCLINICAL PERTUSSIS CASES

Subclinical pertussis infection was diagnosed in 10 infants during the study period. Three of the infants were whole-cell vaccinees and 7 were acellular vaccinees. No study subject had pertussis diagnosed clinically or had a definite history of disease contact. The incidence of subclinical infection was 12.05/1000 doses of whole-cell vaccine and 11.84/1000 doses of acellular vaccine; no significant difference was noted.

9.5.2 SEROLOGY

Whole-cell pertussis vaccination of uninfected age-matched infants in the present study provoked antibody responses against AGG2,3 but not against PT and FHA. Acellular pertussis vaccination resulted in significant PT and FHA antibody titres, but little or no response to AGG2,3 in the study population.

Antibody profiles of the 10 infants who had 'subclinical pertussis', ie. ≥4-fold increase in IgG antibodies to AGG2,3 and to either or both PT and FHA are shown in Tables 9.1 and 9.2 and Figures 9.1 to 9.9. The subclinical pertussis led to stimulation of antibody responses to multiple antigens of *B. pertussis*.

Antibody levels attained were significantly higher for all pertussis antibody assays than those detected in age-matched unexposed vaccinees ($p < 0.001$), and hence could not be attributed to vaccination. Nine of the 10 infants had ≥4-fold increases in all 3 IgG antibodies measured. One infant had ≥4-fold increases in IgG antibodies to FHA and AGG2,3, but not PT.

Pertussis antibody concentrations were measured in serum taken prior to subclinical pertussis infection. These were not significantly lower in children who had subsequent serologic evidence of infection compared with those who did not.

Peak IgG anti-PT levels (at the time of subclinical pertussis) ranged from ≥2.9 to ≤135-fold higher, peak IgG anti-FHA ≥4 to ≤27-fold higher and peak IgG anti-AGG2,3 ≥5 to ≤51-fold higher than those in age and vaccine-matched uninfected infants (Table 9.3).

Serum IgA antibodies to PT and FHA were detected in all 10 of the above infants, 3 of whom had only a very limited rise in IgA antibodies (Table 9.5). IgA responses to AGG2,3 occurred

less frequently than to FHA and PT: 4/10 infants (40%) had demonstrable IgA-anti AGG2,3 antibodies (3 of whom were acellular vaccine recipients).

The ratio of peak to pre-infection geometric mean titres in the 10 infants with subclinical pertussis are shown in Table 9.4.

The scale of the IgG response to infection in the present study in subjects who received one dose of acellular vaccine ranged from >2.5 to ≥135-fold (PT), >5 to ≥27-fold (FHA) and >5 to ≥51-fold (AGG2,3) greater than those recorded in age and vaccine-matched uninfected infants.

Placentally-derived IgG antibodies to FHA, PT and AGG2,3 were present in cord blood of all subjects. Maternal and cord serum IgG-PT, IgG-FHA and IgG-AGG2,3 titres for both infected and uninfected infants are shown in Tables 9.5, 9.6 and 9.7. Cord IgG-AGG2,3 titres were significantly lower ($p = 0.048$) in infected infants than in uninfected infants. All but 1 infant, ie. 9/10, had cord blood anti-AGG2,3 levels which were significantly lower than those of uninfected infants (Table 9.6). The degree of transplacental transfer was not significantly different from that in uninfected infants.

Cord blood anti-PT levels were lower in 8 of 10 of the infants under study than in infants who did not develop subclinical infection and were higher in 2/10 subjects. Cord blood anti-FHA levels were lower than those in uninfected infants in 8/10 subjects, higher in 1 subject, and similar in 1 subject.

9.5.3 AGE AND GENDER

All infants were ≤8 months of age at the time of subclinical pertussis infection; four infants were ≤2 months old, 5 infants were between 2 and 4 months old and 1 infant was between 6 and 8 months old. Infection occurred almost equally in girls and boys (6:4) (Table 9.7).

9.5.4 VACCINATION STATUS

Only 1 of the 10 infants had completed primary vaccination (ie. 3 doses of W-DTP); four infants

Only 1 of the 10 infants had completed primary vaccination (ie. 3 doses of W-DTP); four infants were unvaccinated and 5 had been vaccinated once (4 with A-DTP and 1 with W-DTP, all according to routine vaccination schedules commenced at 2 months of age).

9.5.5 CLINICAL RECORD

Details are presented in Table 9.1. Four of the infants were reported to have had upper respiratory tract infections at or immediately prior to the age of peak antibody response. The percentage of non-infected infants with upper respiratory tract infections was 32.9% below 2 months of age, 37.3% between 2 and 4 months, 38.9% between 4 and 6 months and 37.7% between 6 and 9 months. One infant developed a lower respiratory tract infection at 9 months of age, 7 months after subclinical infection. Other illnesses reported included infections of the skin (9), gastrointestinal tract (5), eye (1) and anaemia (1). Children with serologic evidence of *Bordetella* infection did not experience major post-vaccination events following subsequent doses. Two infants experienced minor symptoms (1 had loss of appetite and excessive crying, and the second excessive crying only).

9.5.6 NUTRITIONAL STATUS

Using NCHS standards, and the anthropometric indices of weight- and length-for-age (Waterlow *et al.*, 1987), subjects were found to be adequately nourished at birth, at vaccination and at the time of subclinical infection.

9.5.7 NUMBER OF CONTACTS WITH STUDY TEAM PRIOR TO PEAK ANTIBODY RESPONSE

Four infants had no contact, 5 had 1 contact and 1 had 3 contacts.

9.6 DISCUSSION

Several investigators have suggested that age, pre-existing illness and immune status may modify the severity of infection and alter the clinical features of pertussis to atypical symptoms

without whooping (Grob, 1981; Cherry *et al.*, 1988; Romanus *et al.*, 1987; *Ad Hoc* Group for the Study of Pertussis Vaccines, 1988).

The majority of infants in the present study were 4 months of age or younger at the time of infection. Most other studies of subclinical pertussis have been in older children and adults. Nutritional status was not found to be an important factor in subclinical disease.

No infected infant had illness with coughing of longer than 1 weeks duration around the time of antibody rise. However 4 infants (40%) were noted to have had signs of mild upper respiratory tract infection at this time which, although not significantly different from the occurrence in non-infected infants, may have been indicative of modified illness. Suppressed clinical expression in these infants may have resulted from partial protection by maternally acquired (present in all) or vaccine-induced (present in some) pertussis antibodies (Preston, 1977).

The current study extends the knowledge of contagious spread of subclinical infection to young infants with high levels of circulating maternally-acquired pertussis antibodies. Variable levels of PT, FHA and AGG2,3 IgG antibodies, ranging from low to high, were detected in maternal and cord sera, confirming findings of Phillips (1921) that neonates are not protected by maternally-derived immunity to pertussis. The presence of maternal antibodies is probably the end result of natural infection (as the currently used whole-cell vaccine produced poor responses to PT and FHA) and indicates that pertussis is widespread in this community.

There is some evidence of the likelihood of transmission of infection from subclinical cases to vaccinated individuals in contact with them in the household, at health services or in the community (Mertsola *et al.*, 1983; Long *et al.*, 1990b). The occurrence and magnitude of subclinical infection is unknown. In the past children with whooping cough were thought to be the source of family spread. Studies now show that most spread is due to older vaccinated persons with modified illness (Nelson 1978; Bass & Stephenson, 1987; Mortimer, 1990). The importance of asymptomatic or mildly symptomatic individuals in silent transmission of

infection is incompletely understood. The spontaneous acquisition of pertussis antibodies in 'inner city' areas where the disease is infrequently recognised is indicative of silent transmission (Long *et al.*, 1990a). Data from this study suggests that *Bordetella pertussis* infection occurs in very young infants who are unvaccinated, incompletely vaccinated, or have had complete primary vaccination. This probably occurred through casual exposure to infected individuals. The source of contact was unidentified. No child had household or other known exposure to anyone with pertussis. Infants may have been infected by unrecognized or subclinical cases in the household, community, or during visits to the health clinic. At least 4 of the infants, who had been enrolled in the study at birth, had not visited the clinic prior to the subclinical episode.

It is known that pertussis vaccination provides better protection against disease than against infection (Long 1990b, Thomas 1989, Mortimer 1990, Fine & Clarkson, 1987). The overall findings in this study, especially the detection of subclinical cases among infants incompletely and completely vaccinated with 3 doses of DTP, are in accordance with this effect of the vaccine.

There is nearly universal agreement that PT is responsible for the clinical manifestations of pertussis and is the essential protective antigen, although there are some case reports of convalescents who lack detectable serum anti-PT (Burstyn *et al.*, 1983). Antibodies to FHA may play a part in protection against pertussis when anti-PT antibodies are also present, but are not essential for recovery. Several studies have found that antibodies to FHA are not uniformly detected in convalescent sera (Winsnes *et al.*, 1985; Nagel *et al.*, 1985). Antibodies to FHA may indicate mild to asymptomatic infection with *B. pertussis* or other Bordetella species, whereas antibodies to PT are produced only by *B. pertussis* (Keogh *et al.*, 1947; Granström *et al.*, 1982; Irons *et al.*, 1983). Anti-PT and anti-FHA are probably the most important antibodies in preventing adherence of *B. pertussis* to respiratory tract epithelium. The role of IgG-AGG2,3 antibodies in immunity to pertussis remains to be resolved, but in all likelihood, is a supportive one. In the present study >90% of infants who succumbed to infection had cord AGG2,3 titres

which were significantly lower than those of uninfected infants. The significance of antibodies to pertussis-specific adenylate cyclase (AC) toxin in protection is not elucidated.

Both natural disease and whole-cell pertussis vaccination provoke serum antibody production against a variety of *B. pertussis* antigens (Thomas *et al.*, 1989b).

Pertussis infection is reported to induce significantly higher IgG-FHA and IgG-PT titres than those observed after either acellular or conventional whole-cell vaccination (Sato & Sato, 1985; Granström 1985; Granström *et al.*, 1985; Winsnes *et al.*, 1985). IgG-PT titres following 2 doses of acellular pertussis vaccine was only 73% of the value attained by convalescent 2 year old Japanese infants (Sato & Sato, 1985). Winsnes *et al.* (1985) found IgG-FHA titres in convalescent sera to be about 10-fold greater than that in whole-cell vaccinees. Furthermore, modest IgG agglutinogen antibody rises have been observed after whole-cell vaccination (Long *et al.*, 1990a)

All 10 infants in the present study had IgG-FHA and PT levels which were significantly higher than those of uninfected cohorts. Furthermore, all infected infants produced substantial IgG-AGG2,3 antibodies in response to infection with *B. pertussis*. Symptomatic infection is characterised by higher anti-PT antibodies, whereas in the asymptomatic infection, antibodies to FHA tended to be higher (Thomas *et al.*, 1989; Long *et al.*, 1990b). Antibody levels in the one symptomatic infant in the present study (data not reported) were in accordance with the above.

The role of serum IgA in pertussis has not been clearly defined. Raised IgA-FHA and PT antibody levels appear to be characteristic of infection and not vaccination, and thus may be used to differentiate between the two (Winsnes *et al.*, 1985). These antibodies may only appear 6-7 weeks after infection and may not develop in all infected individuals (Nagel & Poots-Scholten, 1983). Burstyn *et al.* (1983) and Winsnes *et al.* (1985) reported serum IgA anti-PT in only 25% of infected individuals but not in vaccinees. The majority of infants (70%) in this study produced a definite IgA response to FHA and PT; only 30% produced IgA-AGG2,3.

Some reports indicate that subclinical infections with *B. parapertussis* are common (Lautrop, 1971; Linneman & Perry, 1977). It is unlikely that the IgG antibody responses (and therefore the subclinical pertussis) were induced by infection with *B. parapertussis*, viral infection or nonspecific stimulation of pertussis antibodies. All infected infants had significant rises in antibodies to PT, which are not produced by *B. parapertussis*; furthermore, multiple pertussis antibodies were raised in infected infants and other siblings or babies in the community were not affected by these non-pertussis infections. The alternative interpretation, that these are maternal antibodies, seems unlikely as the titres could not have resulted assuming a half-life of 3 to 4 weeks for maternal antibody.

Vaccine efficacy studies are based on clinically evident cases of pertussis and efficacy rates ranging from nil to 95% have been reported for whole-cell preparations (Fine & Clarkson, 1987). The efficacy of acellular vaccines is currently under investigation. Pertussis infection was diagnosed in only one child prospectively on clinical grounds in the present study. Although not designed to determine the protective effect of the vaccines used (sample size being too small to make a definite connection) the detection of subclinical pertussis infection in this study, highlights the limitations of clinical diagnostic criteria used in developed countries (Fisher *et al.*, 1981; *Ad Hoc* Group for the Study of Pertussis Vaccines, 1988) for disease surveillance and pertussis vaccine efficacy studies, as infection and transmission of the organism may occur in the absence of detectable clinical clues.

The present study adds to the information on obscure causes of infant mortality; subclinical pertussis in the very young may be an unrecognized cause of post- and perinatal mortality and morbidity (Nicoll & Gardner, 1988; Halperin *et al.*, 1989). Classical disease symptoms are rare in very young infants. It is possible that infection may progress to disease with serious pathology in the absence of typical features in such cases and mortality from pertussis may hence be attributed to other causes. The magnitude of the antibody response to subsequent

doses of acellular or whole-cell vaccine was no different in infected and uninfected infants.

Most other evidence of subclinical disease has been in recipients of conventional whole-cell vaccine. Both whole-cell and acellular vaccinees in the present study were affected. However the number of children was small and had to be subdivided for the purposes of analysis according to whether or not vaccinated. Any conclusions drawn can therefore only be very preliminary.

TABLE 9.1 Patient details.

Case*	Sex	No of vaccine doses prior to peak response	Peak antibody response (age in mths)	Post-vaccination events (age in mths)	Clinical problems (age in mths)
1	F	0	2	nil	URTI (2)
2	F	1	4	nil	S (4,6)
3	M	3	8	nil	URTI (6) S (6,9)
4	F	0	2	LOA,C (6)	URTI (2,6)
5	M	0	2	nil	URTI (2) S (0,6,9) GIT (6)
6	M	0	2	nil	LRTI (9) S (6)
7	F	1	4	nil	S (4)
8	F	1	4	C (4)	URTI (2,6) F (2) GIT (9)
9	M	1	4	nil	GIT (6)
10	F	1	4	nil	URTI (4) GIT (6)

* Cases 1-3 Whole-cell vaccinees.
Cases 4-10 Acellular vaccinees (commenced at 2 months).

Abbreviation:

LRTI	- lower respiratory tract infection
URTI	- upper respiratory tract infection
S	- skin infection
F	- fever
GIT	- diarrhoea
LOA	- loss of appetite
C	- excessive crying

TABLE 9.2 Comparison of antibody titres in acellular pertussis vaccinees who had subclinical pertussis (n = 7)* with those who remained uninfected.

		Pertussis IgG antibody levels at time of infection: GMT ± SE (n)	
		>0 ≤ 2 months	>2 ≤ 4 months
PT			
Pre-infection	Cases	35.8 ± 5.4	44.9 ± 8.8
	Non-cases	48.8 ± 11.4 (75)	18.5 ± 3.4 (64)
Peak titre	Cases	380.4 ± 0.2	299.9 ± 10.6
	Non-cases	18.5 ± 3.4 (64)	78.5 ± 9.7 (74)
Final titre**	Cases	580.1 ± 11.9	152.1 ± 4.9
	Non-cases	136.4 ± 15.5 (57)	136.4 ± 15.5 (57)
FHA			
Pre-infection	Cases	28.8 ± 7.0	56.1 ± 11.3
	Non-cases	86.1 ± 10.2 (73)	30.1 ± 3.8 (57)
Peak titre	Cases	245.5 ± 10.8	361.2 ± 0.1
	Non-cases	30.1 ± 3.8 (57)	49.1 ± 4.5 (66)
Final titre	Cases	630.1 ± 42.2	187.1 ± 36.7
	Non-cases	116.9 ± 14.3 (49)	116.9 ± 14.3 (49)
AGG2.3			
Pre-infection	Cases	149.9 ± 8.7	90.2 ± 7.5
	Non-cases	143.7 ± 26.9 (74)	54.3 ± 9.0 (59)
Peak titre	Cases	1426.9 ± 51.8	1084.9 ± 13.6
	Non-cases	54.3 ± 9.0 (59)	54.3 ± 7.9 (66)
Final titre	Cases	602.6 ± 27.5	289.7 ± 32.8
	Non-cases	94.0 ± 22.4 (50)	94.0 ± 22.4 (50)

* At time of infection 3 subjects were ≤ 2 months old and 4 were between 2 and 4 months old.
** At 9 months of age.

TABLE 9.3 Comparison of antibody titres in whole cell pertussis vaccinees who had subclinical pertussis (n=3)* with those who remained uninfected

		Pertussis IgG antibody levels at different times of Infection: GMT ± SE		
		>0≤2 months	>2≤4 months	>6≤8 months
PT				
Pre-infection	Cases	79.4	7.8	6.7
	Non-cases	48.2 ± 9.8 (56)	31.7 ± 10.3 (49)	23.4 ± 6.9 (57)
Peak titre	Cases	484.0	1058.3	239.3
	Non-cases	31.7 ± 10.3 (49)	20.5 ± 6.2 (54)	18.4 ± 4.2 (45)
Final titre**	Cases	61.0	498.1	***
	Non-cases	18.4 ± 4.2(45)	18.4 ± 4.2 (45)	-
FHA				
Pre-infection	Cases	415.6	24.6	9.2
	Non-cases	78.5 ± 15.1 (55)	82.8 ± 46.0 (50)	62.5 ± 15.0 (60)
Peak titre	Cases	2328.9	664.1	241.9
	Non-cases	82.8 ± 46.0 (50)	68.3 ± 20.0 (54)	60.8 ± 12.5 (50)
Final titre	Cases	461.0	357.5	***
	Non-cases	60.8 ± 12.5 (50)	60.8 ± 12.5 (50)	-
AGG2.3				
Pre-infection	Cases	290.3	160.9	82.3
	Non-cases	522.5 ± 290.5 (54)	205.6 ± 94.9 (48)	486.9 ± 110.8 (54)
Peak titre	Cases	5705.4	8275.0	2279.6
	Non-cases	205.6 ± 94.9 (48)	347.5 ± 143.7 (50)	714.3 ± 160.2 (44)
Final titre	Cases	3665.0	2402.1	***
	Non-cases	714.3 ± 160.0 (44)	714.3 ± 160.0 (44)	-

* At time of infection subjects were between 2 and 4 months, between 4 and 6 months and between 4 and 8 months old.
** At 9 months of age.
*** Not done.

TABLE 9.4 Ratio of peak to pre-infection geometric mean IgG antibody titres in ten infants with subclinical pertussis.

CASE	NUMBER OF VACCINE DOSES* (received at the time of infection)	PERTUSSIS IgG ANTIBODY FHA	PT	AGG2,3
1	0	5	6	19
2	1	27	135	51
3	3	26	35	28
4	0	4.8	6.5	5.8
5	0	15.6	11.3	27.8
6	0	5.1	4.1	18.2
7	1	14.5	5.5	6.7
8	1	10.6	15.0	16.0
9	1	2.9	6.1	9.9
10	1	5.2	8.5	9.6

* Case 1-3: whole-cell vaccine
Case 4-10: acellular vaccine.

TABLE 9.5 Pertussis IgA antibodies in 10 infants with subclinical pertussis.

	IgA-PT	IgA-FH	IgA-AGG2,3
Whole-cell vaccine			
Case 1	+	+	-
Case 2*	+	+	-
Case 3	+	+	+
Acellular vaccine			
Case 1*	+	+	-
Case 2*	+	+	-
Case 3	+	+	+
Case 4	+	+	-
Case 5	+	+	+
Case 6	+	+	+
Case 7	+	+	-

* Very limited rise in IgA levels to PT and FHA.

TABLE 9.6 Maternal and cord serum IgG-PT titres in ten infants with subclinical pertussis and in their mothers.

SAMPLE (n)	GMT ± SE*	RANGE	SD	P-value**
Maternal				
Cases (10)	29.7 ± 6.6	14.8-84.6	20.7	t= -1.8065
Non-cases (224)	45.7 ± 5.9	1.2-1036.9	89.0	p= 0.0812
Cord				
Cases (10)	33.5 ± 8.1	7.8-79.4	25.6	t= -1.3595
Non-cases (225)	46.8 ± 5.5	0.4-631.5	82.0	p= 0.1900
Cord/maternal				
Cases (10)	1.2 ± 0.3	0.26-3.24	0.8	t= -1.1146
Non-cases (221)	1.9 ± 0.6	0.01-134.25	9.1	p= 0.2666

* Geometric mean titre ± Standard error

** Unpaired t-test (unequal variance).

TABLE 9.7 Maternal and cord serum IgG-FHA titres in ten infants with subclinical pertussis and in their mothers.

SAMPLE (n)	GMT ± SE*	RANGE	SD	P-value**
Maternal				
Cases (10)	170.2 ± 80.9	16.4-711.1	255.9	t= 1.1193
Non-cases (223)	79.4 ± 6.3	5.5-618.0	93.7	p= 0.2917
Cord				
Cases (10)	119.6 ± 53.6	16.2-461.9	169.5	t= 0.5967
Non-cases (222)	87.1 ± 8.9	4.9-1376.5	132.3	p= 0.5647
Cord/maternal				
Cases (10)	1.1 ± 0.4	0.09-4.2	1.1	t= -0.5730
Non-cases (218)	1.3 ± 0.1	0.08-16.9	1.5	p= 0.5787

* Geometric mean titre ± Standard error
** Unpaired t-test (unequal variance).

TABLE 9.8 Maternal and cord serum IgG-AGG2,3 titres in ten infants with subclinical pertussis and in their mothers.

SAMPLE (n)	GMT ± SE*	RANGE	SD	P-value**
Maternal				
Cases (10)	362.3 ± 169.2	19.6-1630.0	535.2	t= -0.0263
Non-cases (220)	367.3 ± 82.9	0.9-17404.6	1229.2	p= 0.9794
Cord				
Cases (10)	134.7 ± 29.6	27.7-290.3	93.6	t= -1.9827
Non-cases (220)	366.9 ± 113.3	0.9-21249.3	1680.1	p= 0.0486
Cord/maternal				
Cases (10)	1.0 ± 0.2	0.05-1.7	0.6	t= -0.3604
Non-cases (216)	1.1 ± 0.1	0.02-15.5	1.4	p= 0.7239

* Geometric mean titre ± Standard error
** Unpaired t-test (unequal variance).

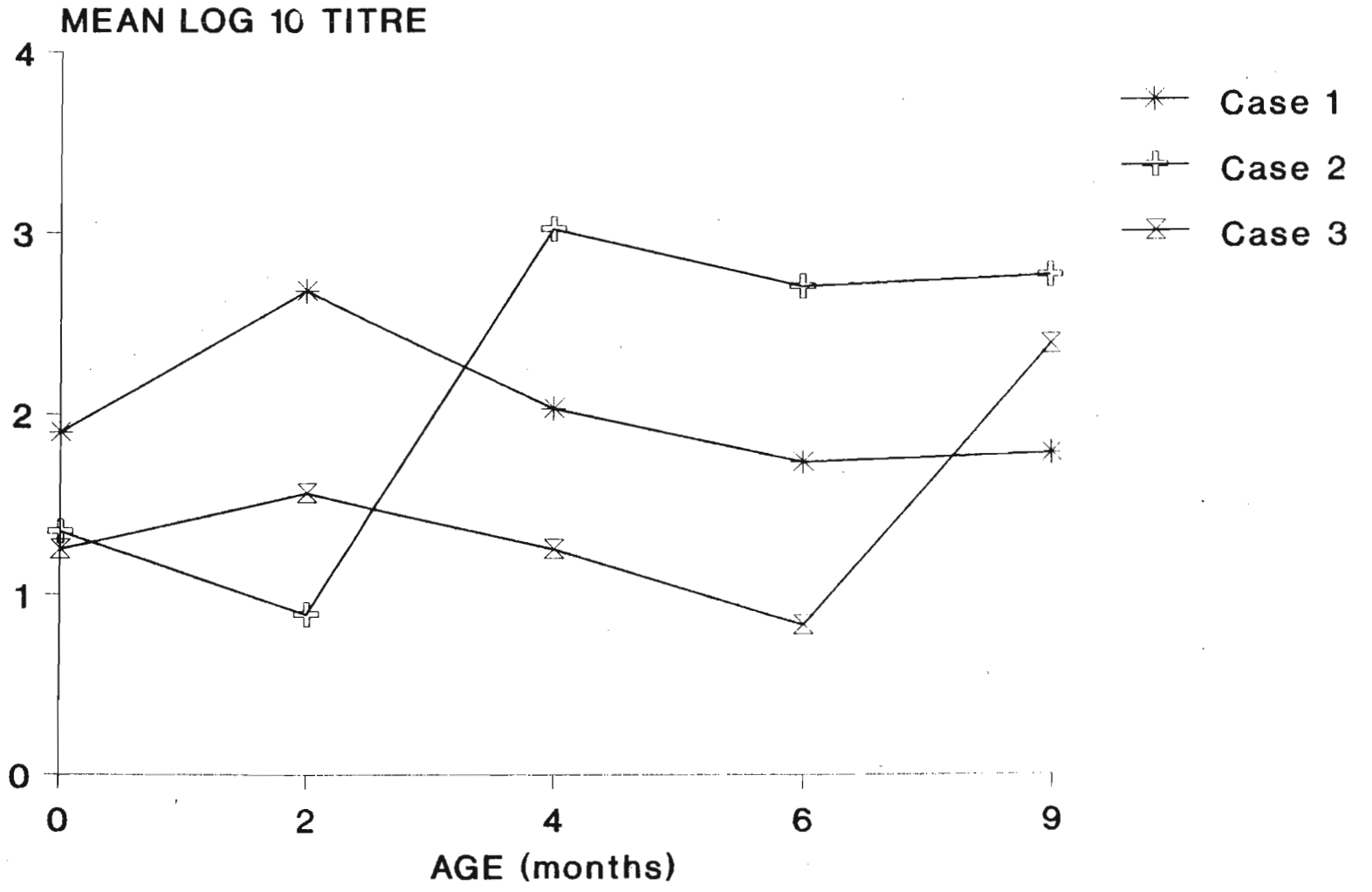


FIGURE 9.1 IgG-PT profiles of 3 whole-cell pertussis vaccine recipients with serologic evidence of pertussis infection.

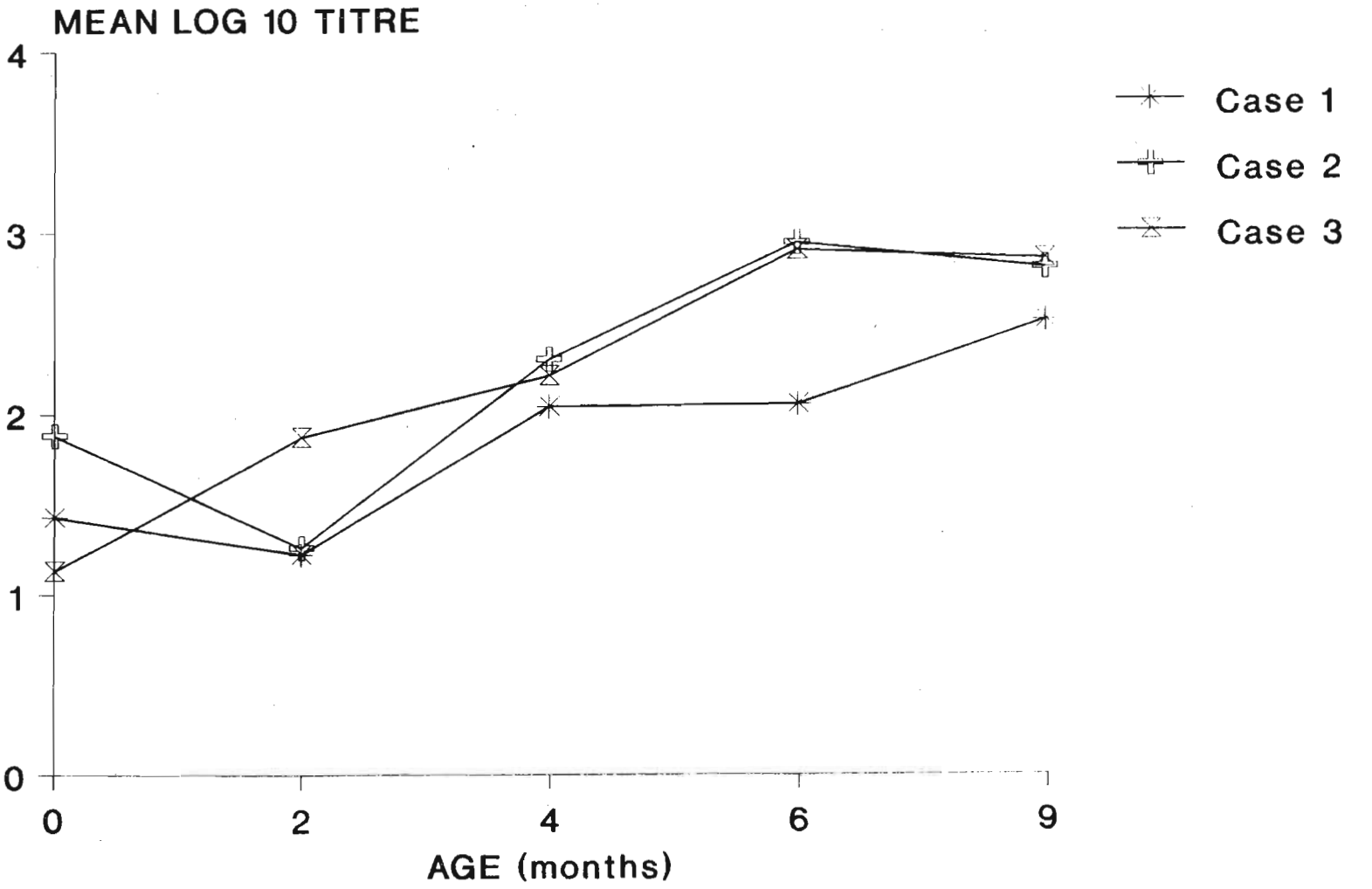


FIGURE 9.2 IgG-PT profiles of 3 acellular pertussis vaccine recipients with serologic evidence of pertussis infection between 2 and 4 months of age.

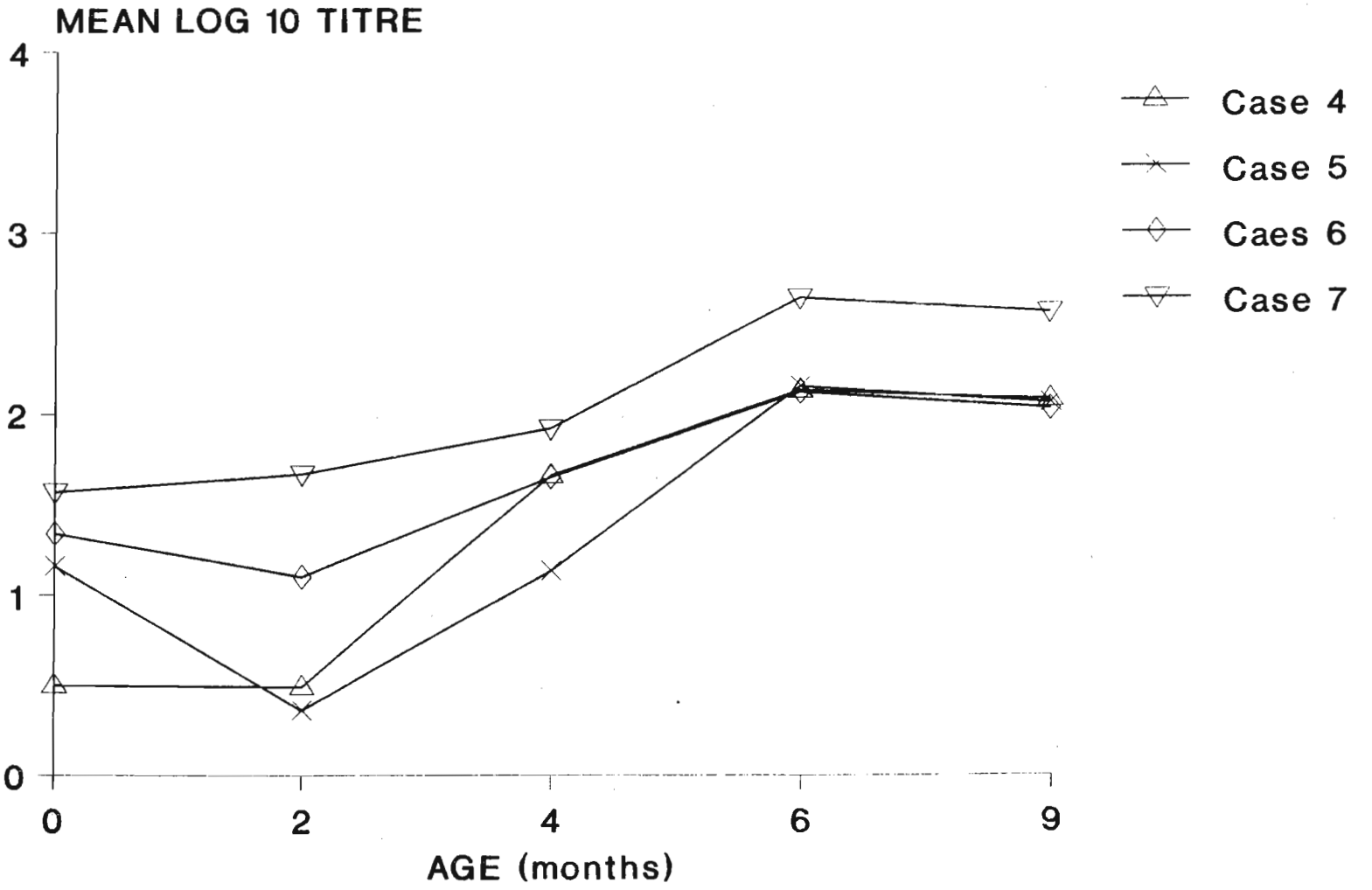


FIGURE 9.3 IgG-PT profiles of 4 acellular pertussis vaccine recipients with serologic evidence of pertussis infection between 4 and 6 months of age.

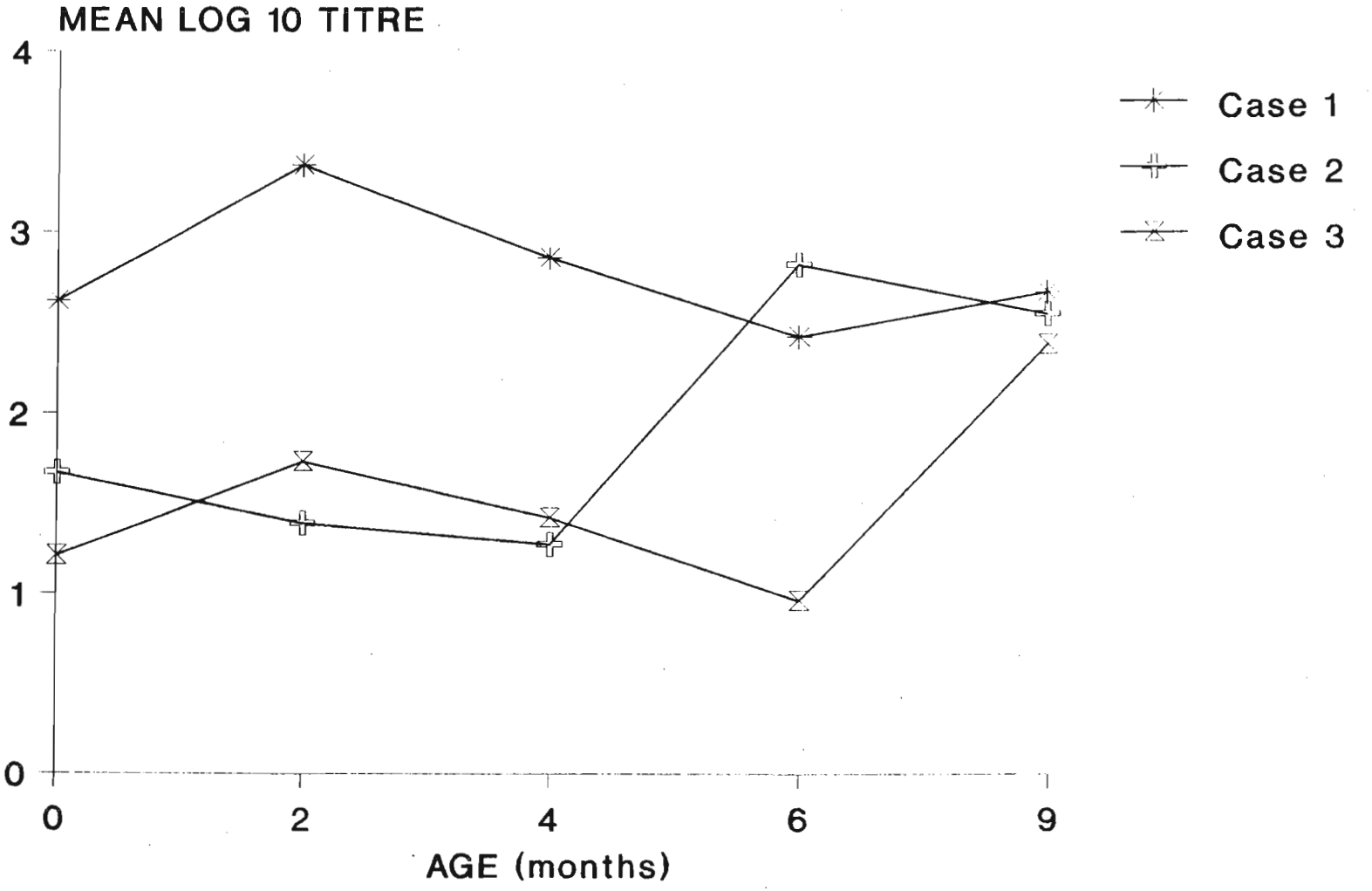


FIGURE 9.4 IgG-FHA profiles of 3 whole-cell pertussis vaccine recipients with serologic evidence of pertussis infection.

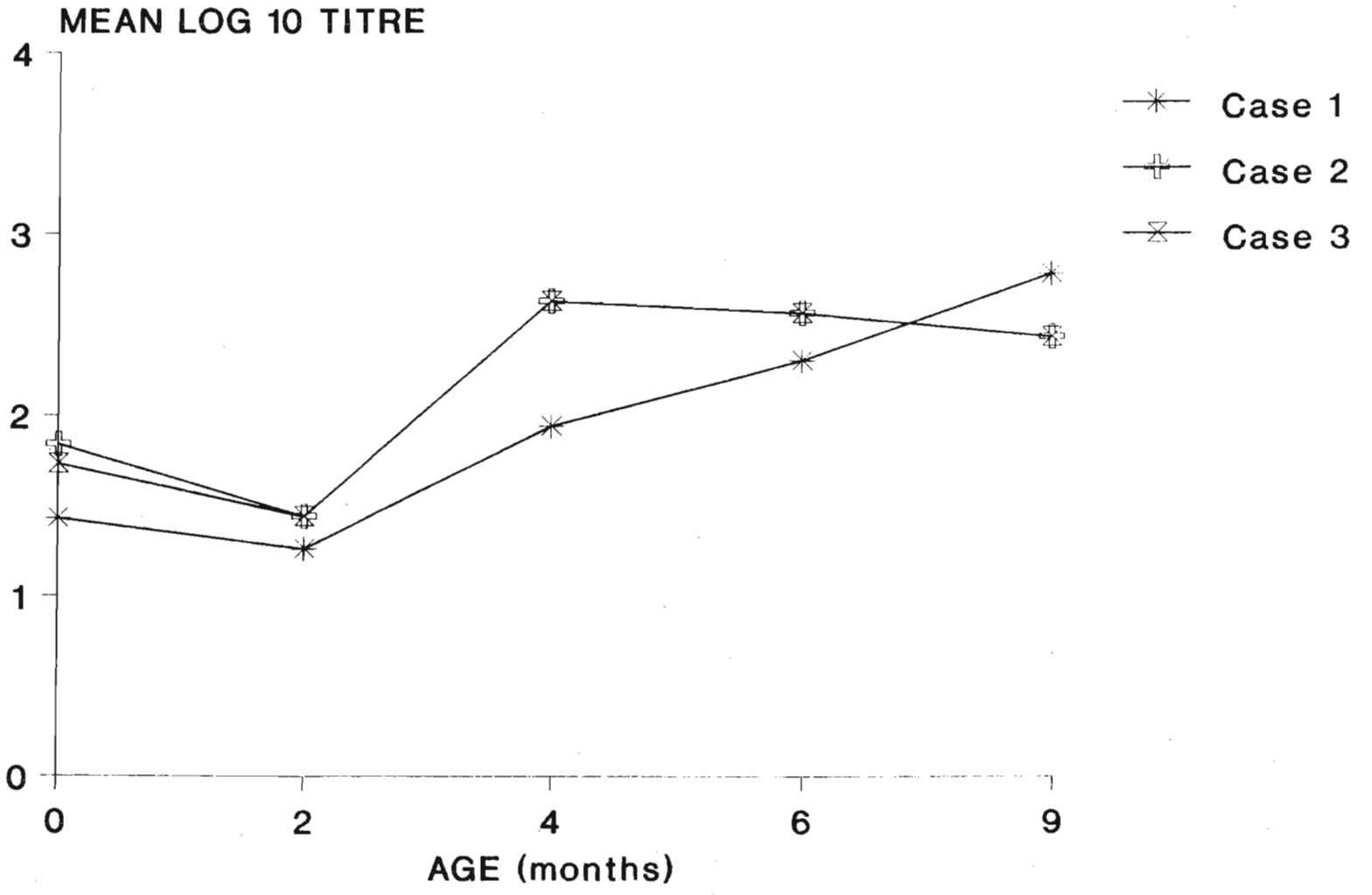


FIGURE 9.5 IgG-FHA profiles of 3 acellular pertussis vaccine recipients with serologic evidence of pertussis infection between 2 and 4 months of age.

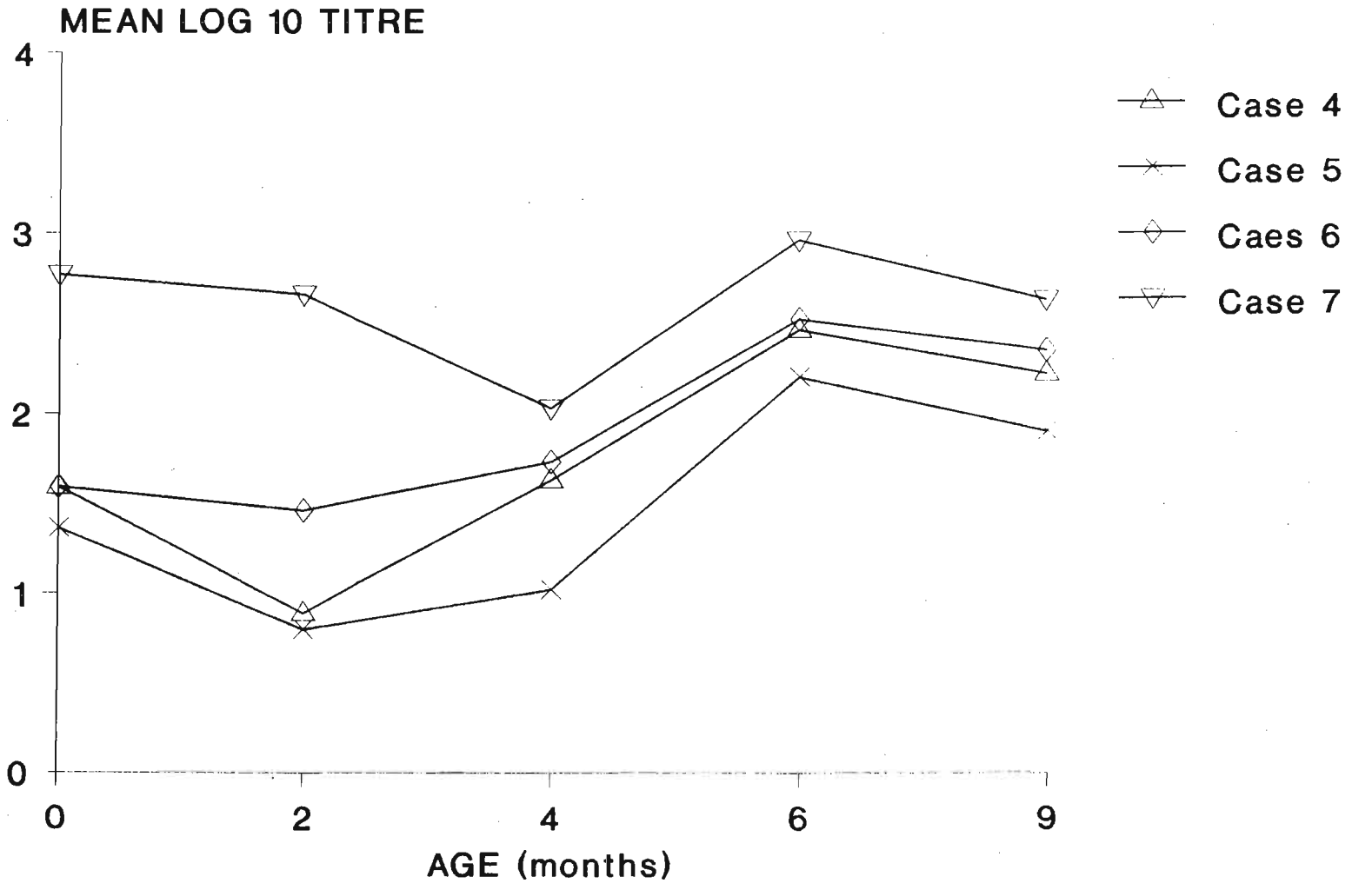


FIGURE 9.6 IgG-FHA profiles of 4 acellular pertussis vaccine recipients with serologic evidence of pertussis infection between 4 and 6 months of age.

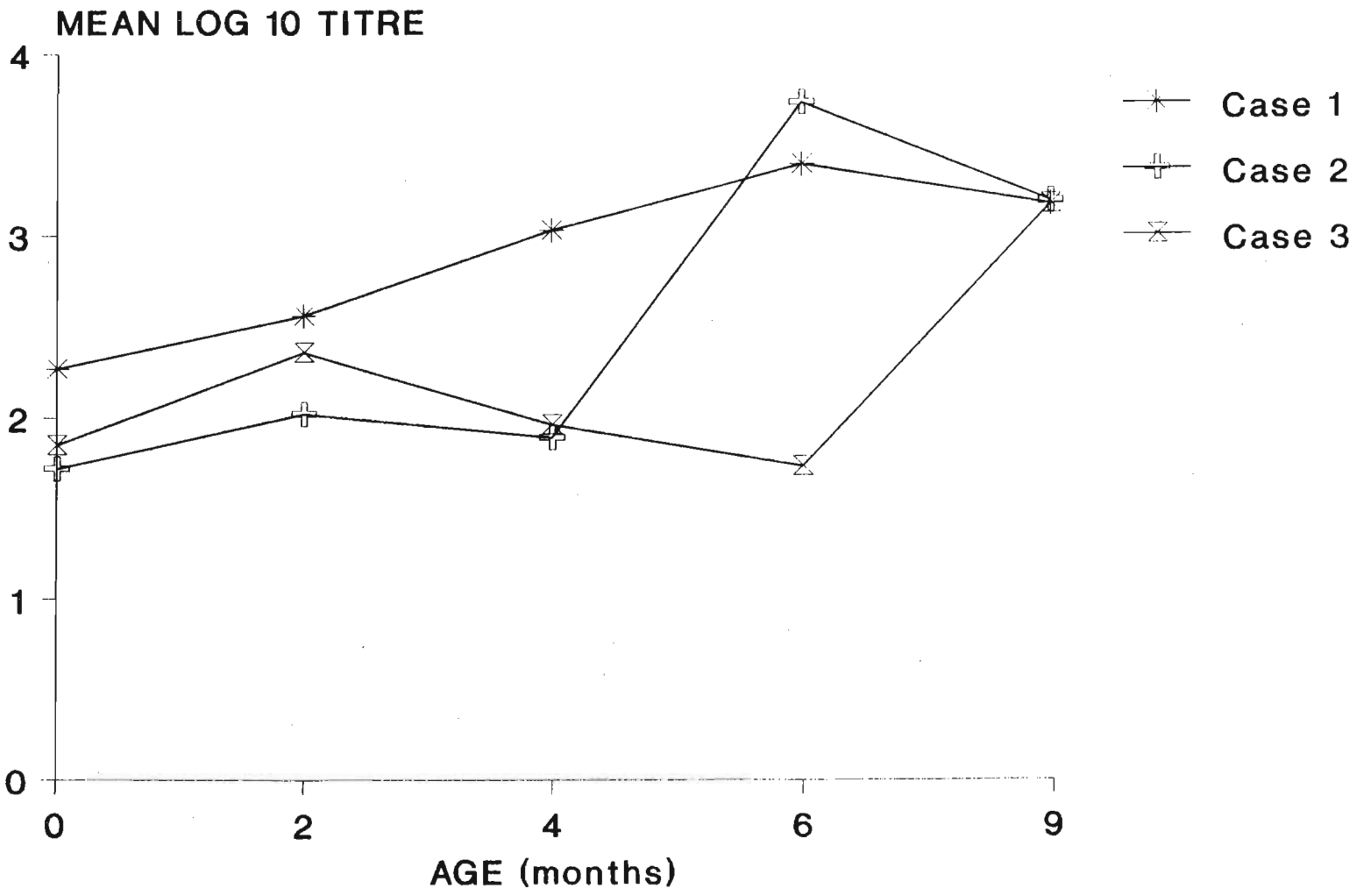


FIGURE 9.7 IgG-AGG2,3 profiles of 3 acellular pertussis vaccine recipients with serologic evidence of pertussis infection.

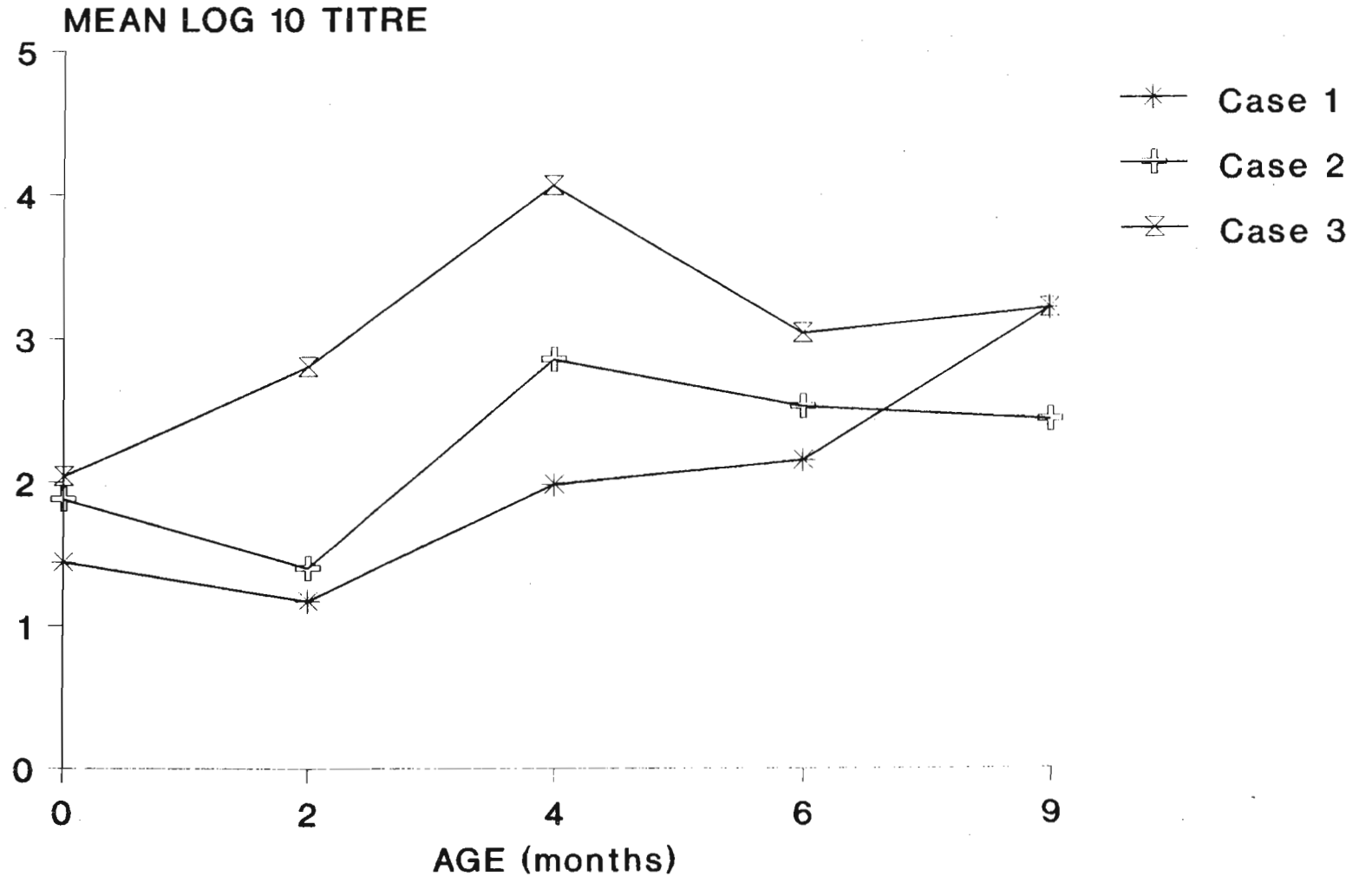


FIGURE 9.8 IgG-AGG2,3 profiles of 3 acellular pertussis vaccine recipients with serologic evidence of pertussis infection between 2 and 4 months of age.

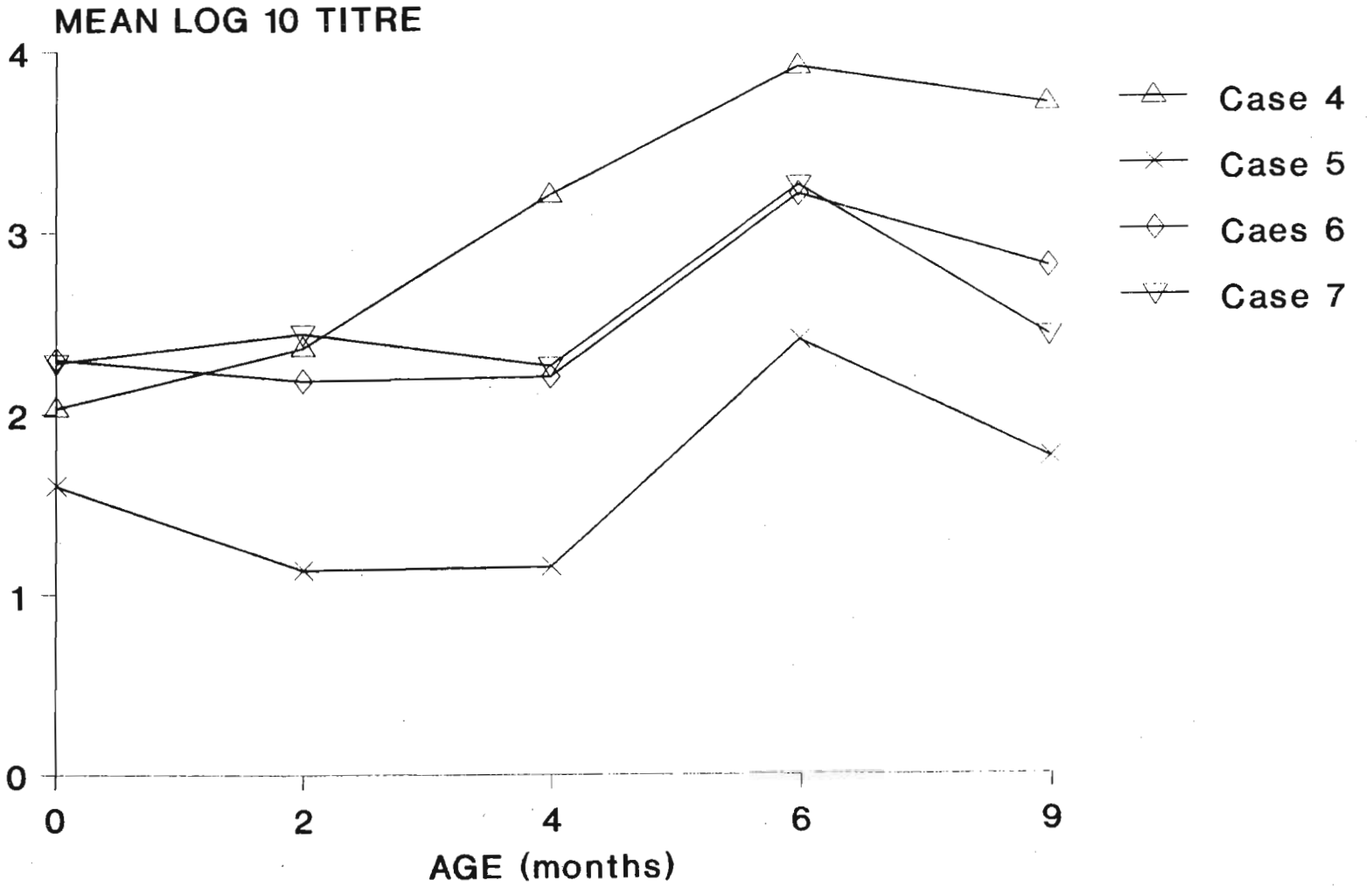


FIGURE 9.9 IgG-AGG2,3 profiles of 4 acellular pertussis vaccine recipients with serologic evidence of pertussis infection between 4 and 6 months of age.

CHAPTER 10

SOME PERINATAL FACTORS AFFECTING VACCINATION

10.1 OBJECTIVES

1. To determine the relative prevalence of maternally-acquired specific IgG antibodies to diphtheria, tetanus and pertussis (DTP) in full-term and pre-term black infants.
2. To evaluate the effect of pre-existing maternally-acquired diphtheria, tetanus and pertussis antibody levels on subsequent serologic response to early and routine DTP vaccination and implications for vaccine efficacy.
3. To determine whether the transplacental transfer of pertussis antibodies (and hence the level of maternally-acquired antibodies) is compromised in small-for-gestational-age, and premature infants and in infants who subsequently develop pertussis infection.

10.2 INTRODUCTION

There are many reports of the occurrence of transplacental transfer of IgG antibodies to *B. pertussis* (Cohen & Scandron, 1943; Kendrick *et al.*, 1945; Adams *et al.*, 1947; Miller *et al.*, 1949; Goerke *et al.*, 1958; Baraff *et al.*, 1984; Marley *et al.*, 1985) to diphtheria toxoid (Nathenson & Zakzewski, 1976; Osborn & Julia, 1952), and to tetanus toxoid (Cates *et al.*, 1988, Einhorn *et al.*, 1987, Sangpetchsong *et al.*, 1984).

The effect of peri-natal malnutrition and prematurity (commonly occurring problems in the Third World) on transplacental transfer of diphtheria, tetanus and pertussis antibodies has not been conclusively established in these areas. Furthermore, there is little known about the nature and rate of pertussis antibody transfer, especially in less developed countries where inhibition of transplacental transfer may occur for a variety of reasons.

Most studies of pertussis antibody transfer have been in developed countries and have shown low antibody titres in infants. Disease prevalence and profile in infants with high levels of maternally-acquired antibody is not adequately elucidated.

10.3 BACKGROUND INFORMATION: DIPHTHERIA, TETANUS AND PERTUSSIS ANTIBODY TRANSMISSION FROM MOTHER TO FETUS

Fcγ receptors in the placenta permit active transfer of specific maternal IgG (Whitelaw & Parkin, 1988), largely in the last trimester of pregnancy. Although a large number of surface Ig-bearing B lymphocytes are evident in the fetus by the 20th week of gestation, negligible amounts of Ig are synthesized. IgG levels in the fetus are extremely low until 20-22 weeks of gestation, at which time active transport of IgG is evident. An acceleration of transfer occurs after the 34-36th week.

IgG concentrations in pre-term infants are directly proportional to gestational age. (Madani & Heiner, 1989). In SGA infants reduced maternal IgG levels may be due to placental dysfunction which may interfere with optimal transfer of IgG across the placenta (Chandra, 1975). In low birth weight infants the capacity to form antibodies may be slightly impaired. Specific antibodies such as tetanus antitoxin are present in lower concentration in the sera of low birth weight (LWB) infants. The fetal/maternal ratio of tetanus antitoxin varies from 0.25 to 1.46, depending upon gestational age and birth weight (Chandra, 1975). Serum antibody titres following Sabin poliovirus vaccine were found to be lower in LBW infants than in healthy full-term controls.

During the fetal and newborn periods most of the IgG in circulation are of maternal origin. Only 7s antibodies pass into the infants, variations in the proportions of these antibodies may account for individual differences in placental transmission. The persistence of passively-acquired antibodies to diphtheria, tetanus and pertussis in the first few months of life is known to correlate with the titre transmitted from the mother, and to the age of the placenta. The titre in a full-term neonate is usually the same or slightly higher than that of the mother (Preston, 1977).

10.4 SUMMARY

The prevalence and nature of maternally-derived IgG antibodies to diphtheria, tetanus and specific *B. pertussis* antigens in full-term, pre-term and SGA infants was investigated. Furthermore, the effect of these maternally-derived antibodies on subsequent serologic responses to early and routine vaccination was investigated in full-term and pre-term (28-37 weeks gestational age) African infants.

Full-term infants were divided into 3 groups. At birth Group I received acellular DTP (A-DTP) and Group II, a saline placebo; Group III received no vaccine. Groups I and II received A-DTP at 2, 4 and 6 months and Group III received whole-cell DTP (W-DTP)

Pre-term infants received DT vaccine at birth followed by W-DTP at 2, 4 and 6 months of age. Term infants served as controls. IgG antibodies to 3 major antigens of *B. pertussis*, PT, FHA and AGG2,3, and to diphtheria and tetanus toxoid (DT) were assayed by ELISA in pre-vaccination sera obtained from mother or infant pairs at birth, and from infants at 2, 4, 6 and 9 months.

The level of pre-existing PT, FHA and AGG2,3 antibodies detected at birth (Group 1) or at 2 months (Groups 2, 3) was found not to affect final antibody titres at 9 months of age to either A-DTP or W-DTP in all groups. Intermediate responses were variable. Infants in Group 1 with low cord anti-FHA and anti-AGG2,3 titres had significantly lower anti-FHA and anti-AGG2,3 at 2 and at 4 months of age respectively than infants with high cord titres ($p=0.004$).

In Group 2, infants with low pre-existing AGG2,3 antibodies achieved significantly lower peak titres at 4 months ($p=0.003$) and at 6 months of age ($p=0.02$) than infants with high titres.

Protective levels of D and T were found in 100% and 76% of cord sera from pre-term infants respectively, and in 85% and 67% of cord sera from full-term infants. There did not appear to

be discernable 'tolerance' due to neonatal DT vaccination or to high levels of maternally acquired antibody.

Although the number of infants was too small to make a definite comment, these observations suggest that the initiation of primary vaccination with DT at birth followed by W-DTP according to routine vaccination schedules at 2, 4 and 6 months need not be delayed beyond 2 months of age in pre-term infants. The above observations are of potential significance to vaccination programmes utilising acellular pertussis and DT vaccines early in infancy.

It was therefore decided that transplacental transfer of diphtheria and tetanus antibodies in malnourished and pre-term infants and in infants who subsequently developed the disease would be investigated. The effects of these transplacentally acquired antibodies on serologic responses to early and routine DT and DTP vaccination was also investigated.

It has become necessary to assess the effect of maternally derived pertussis antibodies on serologic responses to the acellular pertussis vaccines because of the extensive use of these purified agents worldwide, either as replacement for the conventional whole-cell product or in efficacy studies (Sato *et al.*, 1943; *Ad hoc* Group for the Study of Pertussis Vaccines, 1988). This evaluation is especially relevant as there is a shift from the administration of the acellular vaccine in older children to its incorporation into routine immunisation schedules which begin early in infancy (Miller *et al.*, 1991).

There is overwhelming evidence that the commonly used vaccines are effective when given in very young babies despite the presence of pre-existing serum antibodies from the mother. However there are notable exceptions and measles vaccine is the most prominent: the live attenuated measles virus grown in chick embryo fibroblasts and provided in ordinary strength was often ineffective in those under 8 months of age because of maternal antibodies. Recent evidence suggests that this barrier can be overcome by utilizing high titre vaccines (Markowitz

et al., 1990; World Health Organisation, 1990). In this respect, pertussis vaccination has been clouded by the very wide range of efficacies obtained with the whole cell product (Fine & Clarkson, 1987); further, there is lack of clarity because of the relative ineffectiveness of pertussis vaccines when given soon after birth and data indicate an inverse relationship between levels of passive antibody and serologic response to specific antigens in the acellular vaccines (Burstyn *et al.*, 1983). This last observation should be seen in the context of a similar inverse relationship which has been demonstrated for diphtheria and tetanus vaccines successful in early infancy (Osborn & Julia, 1952; DiSant Agnese, 1949; Weibel *et al.*, 1979). Accordingly the question of optimal timing of the first pertussis vaccination remains unanswered although it has been suggested that the whole-cell vaccines be delayed until 3 months of age (Barett *et al.*, 1962; Brown *et al.*, 1964).

The opportunity to analyse some of these relationships arose during two vaccine trials. In the first trial a Japanese acellular pertussis vaccine (J-NIH-6) was compared with the South African whole-cell product (Ramkisoon *et al.*, 1989). In the second trial the effect of neonatal DT vaccination on pre-term infants was investigated. The study design allowed us to investigate -

i. The acquisition of maternal antibodies to *B. pertussis* in paired mother-infant sera from well-nourished full-term, SGA and pre-term infants, and infants who subsequently developed pertussis infection.

ii. The effect of these maternally-acquired antibodies on subsequent antibody responses to acellular pertussis vaccine given soon after birth, and to acellular and whole-cell vaccines administered from the age of 2 months.

iii. The acquisition of maternal antibodies to diphtheria and tetanus (DT) in paired mother-infant sera from full-term and pre-term infants.

iv. The effect of these maternally-acquired antibodies on serologic response to neonatal DT vaccination followed by whole-cell DTP vaccination at 2, 4 and 6 months.

Despite the detection of pertussis antibodies in cord blood, neonates do not benefit from

placentally transferred immunity (Phillips, 1921; Coovadia & Loening, 1988; Forfar & Arneal, 1978; Weichsel & Douglas, 1937). Maternal pertussis IgG antibody has little effect on the epidemiology of the disease and the most significant morbidity and mortality occurs in neonates and infants <6 months of age (Gordon & Hood, 1951).

High levels of pre-existing antibodies (whether maternally-derived or vaccine induced) appear to affect not only serologic responses to vaccination but also the clinical expression of disease.

The role of circulating pertussis antibodies in the prevention of overt or severe whooping cough has been reported in immunised individuals (Long *et al.*, 1990a; Mertsola *et al.*, 1983; Steketee *et al.*, 1978; Fisher *et al.*, 1989). Subclinical pertussis was retrospectively detected on the basis of serologic evidence in 10 full-term and 3 pre-term infants with relatively high pre-infection titres in the present study, most of whom had not completed primary vaccination with acellular or whole-cell vaccine. Findings from these infants were used to examine the relationship of subclinical pertussis to pre-existing maternal antibodies.

Poor seroconversion rates in those with high maternal titres noted in this study are a reflection of initial levels of antibody and do not indicate impaired responsiveness or decrease in absolute quantities of antibody produced. Accordingly, final titres rather than seroconversion rates have been emphasized in this assessment.

The development of purified component pertussis vaccines, which appear to be safer than conventional whole-cell preparations (although optimal vaccine composition remains to be defined), requires that the concept of early vaccination be re-examined in order to optimise the immune response to these vaccines in infants; more especially since neonates do not appear to benefit from passive immunity.

Low vaccination coverage, poor socio-economic conditions, and occasional reported

outbreaks suggest that diphtheria remains an important preventable disease in the Third World. Neonatal tetanus may account for 20% to 70% of the total neonatal mortality in these areas. It has been estimated that for every 100 children born in developing countries, 1 will die of neonatal tetanus (WHO, 1988). In South Africa, approximately 80% of tetanus notifications occur in infants <1 year old.

The occurrence of prematurity and low-birth weight are common in developing countries. These infants are more vulnerable than term infants because they have less IgG (Bernbaum *et al.*, 1984). The fetus obtains most of its IgG transplacentally and there is a linear relationship between IgG levels and gestational age. In the past, vaccination of pre-term infants was often delayed because of concern that the immune system was not sufficiently mature to mount an adequate response to vaccination and that these infants might be more prone to adverse reactions (Lingman *et al.*, 1986).

Available evidence now indicates that the ability to mount an immune response is related to the length of time the infant has been exposed to extra-uterine life, and not to gestational age. An early study showed that the immune response to diphtheria toxoid administration at birth was as good as in pre-term infants of 35 weeks gestational age as in term infants (Dancis *et al.*, 1953).

Satisfactory antibody responses and a low frequency of side effects have been documented in pre-term infants when commonly used vaccines are administered at the recommended chronological age for term infants (Bernbaum *et al.*, 1985; Smolen *et al.*, 1983; Conway *et al.*, 1987; Pullan & Hull, 1989).

The need to achieve early effective protection against diphtheria and tetanus in high-risk infants, eg. prematurity in developing countries where infants may receive little or no passive immunity requires that the concept of early vaccination of pre-term infants be investigated in

order to optimise immune responses. The effect of neonatal DT followed by 3 doses of whole-cell DTP according to routine vaccination schedules at 2, 4 and 6 months on subsequent serologic responses was investigated in infants ranging in gestational age from 28 to 37 weeks.

10.5 SUBJECTS AND METHODS

Details of patients and methods are provided in Chapters 4 and 5; hence only a very brief account is given here in this section so that the chapter may be read as a whole.

10.5.1 STUDY POPULATION

Full-term and SGA infants: Data were collected as part of a large open unblinded study of the antibody responses and post-vaccination events following acellular and whole-cell pertussis vaccination (Chapter 8). In March to May 1988, 345 healthy full-term newborn infants from Kwa Mashu, a peri-urban suburb of Durban, South Africa, inhabited exclusively by blacks, were enrolled in the study and were assigned in sequence of birth to one of three groups of 115 children each, for receipt of either A-DTP (Groups I and II) or W-DTP (Group III), and trivalent oral polio vaccine (TOPV) according to routine vaccination schedules at 2, 4 and 6 months of age. In addition, at birth, all infants received TOPV and BCG; Group I received an additional dose of A-DTP; and Group II, a saline placebo injection. Measles vaccine was administered to all infants at 9 months of age.

Three vaccine-unrelated deaths occurred in infants younger than 2 months of age. All had been assigned to Group III. Of the 342 remaining subjects, 68% returned for scheduled visits at 4 months of age, 58% at 6 months and 51% at 9 months.

Pre-term infants: Data were collected as part of a small study of antibody responses and post-vaccination events following neonatal DT administration soon after birth in 34 pre-term infants. This was followed by conventional whole-cell DTP at 2, 4 and 6 months of age. Infants ranged in

gestational age from 28 to 37 weeks. Only 16 infants (47%) returned at 2 months, 14 (41%) at 4 months, 11 (32%) at 6 months and 6 (18%) at 9 months.

Control group: Serological data in SGA and pre-term infants were compared with that obtained in age- and vaccine-matched full-term AGA infants.

Small-for-gestational-age (SGA): Infants with a birth weight or weight-for-length on or below the 10th percentile curve (Lubchenco *et al.*, 1966).

Full-term infants: Includes all AGA and SGA infants.

Pre-term infants: Infants born with a gestational age ≤37 weeks. Gestational age was assessed according to the Dubowitz score derived from neurological plus external criteria (Dubowitz *et al.*, 1970). Nutritional status was assessed according to a combined intra-uterine neonatal growth chart for height, weight, and head circumference (Lubchenco *et al.*, 1966). This is shown in Appendix 6. Infants were divided into 2 groups on the basis of gestational age (28-32 weeks and 33-37 weeks for the purpose of analyses).

Serum samples were obtained at birth from mothers-infant pairs (full-term) or from infants (pre-term) and from all infants prior to vaccination at 2, 4, 6 and 9 months of age. Sera thus obtained were coded and frozen at -20°C until antibody assays could be performed. Written informed consent was obtained from parents or guardians prior to enrolment. Approval for the trials was given by the Ethics Committee of the University of Natal's Faculty of Medicine.

10.5.2 CLINICAL FOLLOW-UP

Vaccine-associated local and systemic symptoms were recorded on illustrated record sheets by parents or guardians for 14 days after each dose. The record sheet was assessed and evaluated at every clinic visit (2, 4, 6 and 9 months of age). Intercurrent illnesses up to 9

months of age were monitored through monthly home-visits by a study nurse, with special attention being paid to nutritional state, common childhood infections, and pertussis contacts and symptoms. Furthermore, all infants underwent physical examination and assessment of nutritional indices by a paediatrician at birth and at every clinic visit.

10.5.3 VACCINES

Acellular DTP: This vaccine was the BIKEN B-type (J-NIH-6) adsorbed preparation from a single batch. One dose of acellular vaccine contained 28 Lf units of diphtheria toxoid, 7 Lf units of tetanus toxoid, 7.5 μg each of purified PT and FHA and 0.075 mg aluminium phosphate in phosphate-buffered saline (PBS) with 0.01% (w/v) thiomersal.

Whole-cell DTP: This vaccine was supplied as a single lot from the South African Institute for Medical Research, Johannesburg. One dose of whole-cell vaccine contained 25 Lf units of diphtheria toxoid, 6 Lf units of tetanus toxoid, 10,000 x10^6 *B. pertussis* organisms and 1.25 mg aluminium phosphate in PBS with 0.01% (w/v) thiomersal.

Diphtheria-tetanus toxoids (DT): South African adsorbed DT vaccine was manufactured by the SAIMR, Johannesburg, South Africa. It contained purified toxoids adsorbed onto aluminium phosphate and preserved with 0.01% thiomersal.

Injections were administered in the left anterolateral thigh in 0.5 ml doses. Saline placebo and A-DTP were administered via the subcutaneous route, and W-DTP via the intramuscular route.

10.5.4 TRANSPLACENTAL TRANSFER OF ANTIBODIES

This was estimated for all 3 pertussis antibodies (PT, FHA, AGG2,3) and for diphtheria and tetanus antibodies from measurements obtained in full-term (including SGA) and pre-term infants and their mothers.

The formula used was: $T = \frac{Abb}{Abm}$

where, T = placental transfer
Abb = antibody titre in baby
Abm = antibody titre in mother

10.5.5 PERTUSSIS INFECTION

'Subclinical' pertussis was defined as serologic evidence of infection, ie. a significant increase in value of IgG antibodies to AGG2,3 and to either or both PT and FHA between 2 consecutive serum samples taken 8-12 weeks apart, in the absence of the typical clinical picture of pertussis.

Pertussis was diagnosed on clinical grounds on observation (or recent history) of paroxysmal cough, whoop, or cough with associated vomiting, cyanosis, apnoea, subconjunctival haemorrhage, epistaxis or periorbital oedema.

Sera from mother-infant pairs were analysed for specific pertussis antibodies (PT, FHA, AGG2,3) that may enhance protection against pertussis in infants who did not develop the disease, and in infants who developed infection before the age of 9 months.

10.5.6 SEROLOGIC ASSAYS

Serum IgG antibodies to FHA, PT, AGG2,3, and diphtheria toxoid were assayed by micro-ELISA at the Centre for Applied Microbiology and Research, Public Health Laboratory Services, Porton UK by the author. The ELISA procedure used for pertussis antibodies was a modification of that described by Rutter *et al.* (1988). ELISA for antibodies to diphtheria anti-toxin was after the method of Camargo *et al.*, 1984. Concentrations of antigens and conjugates used were determined by checkerboard titrations. FHA and PT for use as antigens in the assays and Reference Pertussis Anti-serum (Human) were obtained from the Research Foundation for Microbial Diseases of Osaka University Japan. Reference diphtheria serum was obtained from

the National Institute of Biologic Standards and Control, UK. The unitage of the test serum relative to the reference sera was calculated by means of parallel-line assays.

Serum IgG antibodies to tetanus toxoid (TT) were assayed by micro-ELISA at the Department of Paediatrics and Child Health, University of Natal as described elsewhere (Conrad & Mbhele, 1980). Human tetanus reference serum was provided by the Natal Blood Transfusion Service, South Africa.

All sera from one individual were tested in the same assays on the same day. In some cases the quantity of serum was not sufficient to carry out all the tests required and therefore the number of samples giving rise to the data shown in the tables are not uniform.

CALCULATION OF RESULTS

Pertussis antibodies: No protective levels have been defined for the antibodies to pertussis antigens. The titre of pre-vaccination maternally-derived antibodies in Group I was the level in cord blood and that for Groups II and III, in pre-vaccination samples taken at 2 months of age. Full-term infants were divided on the basis of their pre-vaccination antibody levels into 'high' (>40 U/ml) and 'low' groups (≤40 U/ml) groups for PT and FHA (Burstyn *et al.*, 1983) and above and below the median titre of maternal titres (90 U/ml) for AGG2,3 antibodies. The median titre was chosen as a cut-off after cord blood data was statistically analysed by a univariate procedure, the data plotted, and quantiles defined as follows -

Quantiles:

100%	Maximum	2585.1	99%	2585.1
75%	Quantile3	205.9		875.7
50%	Median	89.0		470.2
25%	Quantile1	42.5		21.1
0%	Minimum	0.9		12.1
				0.9
Range:		2584.2		

Quantile3-Quantile1:	163.4
Mode:	0.9

Diphtheria and tetanus antibodies: The optical densities of the tetanus reference serum dilution series on each microtitre plate was subjected to logistic analysis. The tetanus antibody titre of each test serum was determined by inverse prediction using the curve generated by the reference series on the same plate. Results were expressed as IU/ml. The limit of testing was ≥ 0.01 IU, the lowest presumed protective level of tetanus antitoxin (DiSant Agnese, 1948,1949; McComb & Trafton, 1950; Ipsen, 1946; Gold *et al.*, 1973; Nelson *et al.*, 1978; Crossley *et al.*, 1979; Viljanen & Nieminen, 1980). The minimum protective level of diphtheria antibodies was ≥ 0.02 IU/ml. A titre of < 0.01 IU/ml was considered negative for both D and T antibodies.

10.6 STATISTICAL ANALYSIS

Calculations on the analysis of antibody titres and ELISA values were performed on logarithmically transformed data. Geometric mean antibody values and standard error (GMT ± SE) were calculated. Infants with probable subclinical pertussis were excluded from calculations of serologic responses to vaccination. The significance of the difference between antibody values for the groups was calculated by the Student's t-test (paired), using 2-tailed probability. Seroconversion in 'high' and 'low' titre pertussis antibody subgroups were compared by Fishers' exact test (2-tailed) or chi-square; depending on cell size. Cord blood titres in the groups were compared using Duncans Multiple Range Test which controls the type I comparison error rate. Statistical significance was designated at $p < 0.05$ (two-tailed). Post-vaccination events in pre-term infants were analysed by 2x2 chi square contingency tables with the various symptoms recorded as present or absent.

10.7 RESULTS

10.7.1 TRANSPLACENTAL TRANSFER OF PERTUSSIS ANTIBODIES

10.7.1.1 Full-term appropriate-for-gestational-age (AGA) infants: Substantial quantities of all three antibodies tested were present in maternal sera and were often detected in higher quantities in cord sera indicating active transfer across the placenta (Tables 10,1-10.3). No significant difference was noted between maternal and cord titres in all 3 vaccine groups for all 3 antibodies (Duncans' Multiple Range Test). The GMTs of PT, FHA and AGG2,3 in cord blood were 46.8 ± 5.5 (n=255); 87.1 ± 8.9 (n=222) and 236.7 ± 113.3 (n=220) respectively. The proportion of infants with 'high' titres of maternally derived PT, FHA and AGG2,3 antibodies was 39%, 44% and 50% respectively. Cord blood PT and FHA antibody titres were not significantly different in neonates of all three vaccine groups. The AGG2,3 titre was found to be slightly higher in recipients of W-DTP compared with A-DTP recipients (p=0.08).

10.7.1.2 SGA infants: Maternal and cord pertussis IgG-PT, FHA and AGG2,3 levels in SGA infants are shown in Tables 10.8-10.10 and Figure 10.7. 53% (8/15) SGA infants had cord anti-PT and anti-FHA titres which were lower than the titres of their maternal serum. 52% of AGA infants had cord anti-PT titres higher than that of their mothers and 48% had core anti-FHA titres higher than that of their mothers. 35% of AGA and 33% of SGA infants had AGG2,3 antibody titres higher than that of their mothers. Although there was considerable variation in maternal and cord antibody levels, the majority of mothers had significant levels of all three antibodies. Only 5% (14/282) of mothers had little or no anti-PT, 0.4% (1/283) no anti-FHA and 1.1% (3/277) no anti-AGG2,3.

10.7.1.3 Pre-term infants: Maternal and cord pertussis IgG-PT, FHA and AGG2,3 levels in pre-term infants are shown in Tables 10.11-10.13 and Figure 10.10. In infants of 33-37 weeks gestational age, cord IgG-AGG2,3 GMTs were significantly lower than the GMT of maternal serum (p=0.0485) (Table 10.20).

10.7.2 TRANSPLACENTAL TRANSFER OF DIPHTHERIA AND TETANUS ANTIBODIES

Maternal and cord IgG diphtheria and tetanus antibody levels in pre-term and full-term infants are shown in Table 10.4 and 10.15 and in Figure 10.13. In cord sera, 100% and 76% of pre-term infants had protective levels of anti-diphtheria and tetanus antibodies respectively. 85% and 67% of full-term infants had protective levels of antibodies to diphtheria and tetanus toxoids respectively.

10.7.3 POST-VACCINATION EVENTS

Full-term infants: Details of events are given in chapters 7 and 8.

Pre-term infants: Four minor symptoms were recorded; 1 infant experienced excessive crying at 2 months of age after the first W-DTP injection, 1 infant experienced swelling at injection site at 4 months (second W-DTP injection), 2 infants incurred 1 symptoms each (swelling, fever) at 6 months after the third W-DTP injection.

10.7.4 CLINICAL RECORD

Details of age-related incidence of intercurrent illnesses, infections and clinical signs in full-term and pre-term infants from birth to 9 months of age are given in Chapter 4.

10.7.5 PERTUSSIS INFECTION

Maternal and cord pertussis IgG-PT, FHA and AGG2,3 levels in 10 full-term infants with subclinical pertussis are shown in Table 10.16-10.18 and Figure 10.9. Cord anti-AGG2,3 titres were significantly higher in infants who did not contract the disease, than in infants who did ($p < 0.05$). Patient details of 4 pre-term infants with subclinical pertussis are shown in Table 10.19. IgG-PT, FHA and AGG2,3 antibody profiles in pre-term infants with subclinical pertussis are shown in Figures 10.10-10.12.

10.7.6 SEROLOGIC RESPONSES TO ACELLULAR AND WHOLE-CELL VACCINATION

FULL-TERM INFANTS:

GMTs at 9 months: There was no significant differences at 9 months of age in the GMTs of PT, FHA and AGG2,3 antibodies between 'high' and 'low' groups for all the children studied. Significant differences detected during the intervening period are given in Tables 10.4-10.6 and Figures 10.1-10.6.

Antibodies to FHA: Infants in Group I (neonatal A-DTP) with 'low' cord IgG-FHA achieved significantly lower GMTs at 4 months of age after 2 doses of A-DTP, than infants with 'high' cord titres. [60.8 ± 9.3 U/ml (n=26) versus 118.4 ± 26.2 U/ml (n=41); p=0.04].

Antibodies to AGG2,3: In Group I, AGG2,3 GMTs at 2 months of age after 1 dose of A-DTP, were significantly different between 'high' and 'low titre subgroups [54.8 ± 12.3 versus 168.9 ± 35.6 (p=0.004)]. In Group II, infants with 'high' GMTs achieved significantly higher GMTs after 2 doses of A-DTP at 6 months of age, than infants in the 'low' titre subgroup [120 ± 20.1 U/ml (n=36) versus 70.3 ± 15.4 U/ml (n=16); p=0.05].

Seroconversion to pt, fha and agg2,3 by 9 months of age: Seroconversion rates to PT, FHA and AGG2,3 are shown in Table 10.7. PT seroconversion was significantly higher in the 'low' titre subgroup than the 'high' titre subgroup in recipients of A-DTP (Groups I and II) (81% versus 33%, and 79% versus 42% respectively); but not in recipients of W-DTP (Group III). FHA seroconversion was significantly higher in 'low' titre subgroups in recipients of both A-DTP and W-DTP; 56% versus 7%, 85% versus 17% and 38% versus 0% in Groups I, II and III respectively. Seroconversion to AGG2,3 was significantly higher in infants with 'low' pre-existing GMTs in Groups I, II and III (65% versus 15%, 31% versus 5% and 89% versus 58% respectively).

Pre-term infants: Pre- and post-vaccination GMTs of antibody to PT, FHA and AGG2,3 for the different gestation groups are shown in Table 10.20. The number of infants at 6 and 9 months

of age was too small to allow evaluation of seroconversion or statistical analysis. The GMT of IgG-FHA at 2 months was significantly lower than the pre-vaccination GMT in pre-term infants of 33-37 weeks gestational age ($p = 0.279$).

10.7.7 SEROLOGIC RESPONSES TO DT VACCINATION

Pre- and post-vaccination GMTs of diphtheria and tetanus antibodies following whole-cell DTP vaccination of pre-term and full-term infants are shown in Table 10.21. The number of infants at 6 and 9 months of age was too small to allow statistical evaluation, or to evaluate seroconversion.

10.8 DISCUSSION

In this report the relative prevalence of antigen-specific antibodies to *B. pertussis* in paired maternal/infant sera is described in healthy well-nourished full-term infants, small-for-gestational-age infants, and infants who subsequently developed subclinical pertussis infection. Furthermore, the effect of 'high' and 'low' maternally-derived pertussis antibodies on serologic responses to acellular and whole-cell pertussis vaccines was investigated in full-term infants. The relative prevalence of maternally-acquired diphtheria and tetanus (DT) antibodies in pre-term and full-term infants and the effect of these antibodies on neonatal DT vaccination is also reported.

This study has demonstrated that high titres of maternally derived circulating B. pertussis antibodies do not have an inhibitory effect on the subsequent serologic response to acellular pertussis vaccine administered in early infancy according to the WHO recommended vaccination schedule, or with the first dose given soon after birth. High pre-vaccination titres of antibodies to PT, FHA and AGG2,3 detected at birth or at 2 months of age, did not depress the mean peak titres or final titres at 9 months of age of all 3 antibodies, following vaccination with either acellular or whole-cell pertussis vaccines. These findings may be of relevance to vaccine

efficacy studies commencing during infancy, as the unrestricted antibody responses were to the principle antigens of *B. pertussis* which are believed to be related to protective immunity (Sato & Sato, 1985). It follows therefore that the titre of mothers' antibody in the infant is unlikely to present a significant barrier to the efficacy of acellular vaccines incorporated into routine vaccination schedules. However, the final antibody level reported here was measured at 9 months of age and longer term studies will be necessary to assess the durability of these antibodies. These results are particularly applicable to developing countries where pertussis vaccines have to be given early in infancy as the disease occurs frequently at this age and morbidity and mortality are highest (Ramkissoon *et al.*, 1989).

It appears that the ability of high titres of passive transplacental antibody to quench serological responses to specific major antigens may have to be assessed for individual vaccines. Varying reactions may be due to whether a killed or live attenuated organism is used, the type and quality of component antigens, the avidity and strength of antibodies produced, the importance of cellular immunity and the relative immunological immaturity of the infant. The findings for pertussis in this report are different to those reported for some other vaccines; impaired antibody responses have been reported to measles vaccine (Albrecht *et al.*, 1977; Reilly *et al.*, 1961), diphtheria toxoid (Osborn & Julia, 1952; DiSant Agnese, 1949), and mumps and rubella vaccines (Weibel *et al.*, 1979). Despite these observations, high levels of pre-existing antibody to diphtheria and tetanus toxoid have not appeared to reduce the efficacy of vaccinating from 2 months of age, or from birth (as was found in the current study) probably because final low levels of antibody are still above the protective threshold or they are boosted by revaccination (Cates *et al.*, 1988; Dengrove *et al.*, 1986).

DiSant Agnese (1949) and Gaisford *et al.* (1960) showed that the majority of young infants vaccinated with DTP beginning in the first week of life subsequently developed protective antibody levels. In the current study it was also demonstrated that high levels of antibody could be achieved in infants given an early neonatal dose of DT. These results are however only very

preliminary given the small number of infants in the study.

In addition to a general immaturity of the immune system, another potential immunosuppressing factor in the newborn is the level of passively acquired maternal antibody. Although the mechanism of this suppression is not clear, Wilson & Miles (1955) postulated that passively acquired antibody could bind to antigen forming a complex. Therefore, transplacentally acquired antibodies might diminish the primary immune exposure. Although no studies exist specifically for tetanus toxoid (TT) a suppressive effect of high maternal antibody level on infant immune response to diphtheria toxoid has been shown (Gaisford *et al.*, 1960; Smith, 1960). However, this effect on diphtheria immune responses is incomplete and can be overcome with multiple antigenic stimuli.

The concept of immunological immaturity of the newborn is closely linked with that of immunological tolerance. While there are studies of tolerance reported for pertussis vaccination (Baraff *et al.*, 1984; Provenzano *et al.*, 1965) there are little data regarding D and T, especially in third world countries where a sizable proportion of newborns have no antibody and are susceptible to tetanus neonatorum.

The ability of pre-term infants to develop diphtheria antibodies was found to be a function of the duration of extrauterine existence and not maturity (Osborne *et al.*, 1952). In full-term infants with high levels of maternally-acquired diphtheria antibody, no differences in final titre were achieved although early titres were low (Osborne & Julia, 1952). Protective levels of diphtheria and tetanus antitoxins were found in 64% and 38% of cord sera from Black infants in New York (Natheson & Zakzewski, 1976).

The first DTP vaccination was not found to be sufficiently immunogenic to initiate significant antitoxin production in infants without maternal antitoxin or in older children (Nelson *et al.*, 1978). The serological response to tetanus vaccination may be affected by various factors, eg.

infection, malnutrition, often present in developing countries. Whereas some workers have found that malnutrition lowers the serological response to TT (Paul *et al.*, 1979), others have found no statistically significant effect (Ghosh *et al.*, 1980; Kielmann, 1972).

Mumps and rubella are usually not given under a year of age, but when administered at 9 months they produce adequate antibodies (Schoub *et al.*, 1989). In the case of measles, the relative ineffectiveness of conventional vaccines given under the age of 8 months, when a significant proportion of infants have persisting transplacental antibody, led to the development of more effective products (high titre vaccines, attenuated virus grown in human diploid cells) (Markowitz *et al.*, 1990).

In the evaluation of new conjugated vaccines against *Haemophilus influenzae* type b (Hlb), the role of high titres of passive antibody in subsequent serological responses has been recently reported (Claesson *et al.*, 1989; Parke *et al.*, 1991). The results obtained are inconsistent: there was an inverse relationship between maternal antibody and serological response to Hlb TT among Swedish infants but not among American infants.

Burstyn et al. (1983) have documented an inhibitory effect of high titres of cord blood serum IgG PT antibodies on the response to whole cell pertussis vaccine; FHA antibody responses were not affected by maternal antibody titres. They believe that "...such antibodies may adversely affect the ability of an infant to respond to immunisation with pertussis vaccine" and that these observations are "...of potential significance to immunisation programs or attempts to eradicate pertussis". This negative effect of maternal antibody, in contrast to the findings in our study might be due to the different vaccines employed. The evaluation of the whole cell vaccine from the data given here is bedeviled by the unexpected overall poor immunogenicity of the batch of the South African product used in the current study. Further, acellular pertussis vaccine, when administered at birth, produced less favourable PT and FHA titres than when vaccination commenced at 2 months of age (Ramkissoon *et al.*, 1989).

In general, although infants under 6 months of age are able to respond to vaccination seroconversion rates and titres increase with successive doses of vaccine and are higher with increasing age at the time of vaccination, presumably because of 'blocking' by maternal antibody in a proportion of younger infants (DiSant Agnese, 1949; Sako *et al.*, 1945; Adams *et al.*, 1947; DuPan, 1958; Miller *et al.*, 1949).

There are varying reports of relative antibody levels in paired maternal-infant sera. It is conventionally understood that there is low grade transplacental transfer of maternal pertussis antibodies (Brown 1960; Morse, 1968; Granström *et al.*, 1982; Biritwum *et al.*, 1985; Kassim *et al.*, 1989; Muller *et al.*, 1988; Goerke *et al.*, 1953). On the other hand relatively high rates of transplacental pertussis antibody activity have also been reported. Thomas *et al.* (1989) detected IgG to FHA, PT and AGG2,3 in serum from all 139 infants tested before the administration of the first DTP vaccine.

The current study showed relatively high levels of transplacental antibody activity in the majority of mother-infant pairs. Individual differences in placental transmission, ie. irregular transmission has been reported in earlier studies (Goerke *et al.*, 1953), Inhibition of placental transfer occurred in a few cases and may be attributable to a variety of reasons, including maternal nutrition, placental abnormalities, low birth weight which require further exploration.

Several studies have reported little or no demonstrable antibodies in women of child-bearing age, most of whom were vaccinated as children (Brown, 1960; Biritwum *et al.*, 1985; Kassim *et al.*, 1989; Burstyn *et al.*, 1983; Muller *et al.*, 1986). Surprisingly, a large proportion of infants in the present study had 'high' titres of maternally derived PT, FHA and AGG2,3 antibodies (39%, 44% and 50%) respectively. Data from this study suggests that *Bordetella* infection occurs frequently among child-bearing women in the black community (given the lack of response to PT and FHA in recipients of South African whole-cell DTP (Ramkissoon *et al.*, 1991) and that newborns acquire considerably high levels of passively acquired *B. pertussis* antibodies. A

history of clinical pertussis in the mother and/or of her having received pertussis vaccine in infancy or early childhood does not however correlate with the presence or level of antibodies in the sera of neonates (Wilkins *et al.*, 1971).

Even though our understanding of pertussis immunity is imperfect, antibodies appear to be important. Final antibody titres are more likely to be related to vaccine efficacy rather than seroconversion rates, in the context of high and low levels of transplacental antibody.

TABLE 10.1 Comparison of pertussis IgG-PT antibody titres in paired maternal and cord sera in full-term infants (ELISA U/ml).

	N	GMT ± SE*	Range	Paired t-test** (cord-maternal)
Group I				
Maternal	93	42.7±5.7	1.2-279.5	t=0.33;
Cord	94	443.0±71.9	0.4-631.5	Prob> | t | =0.7404
Group II				
Maternal	76	52.5±15.4	1.7-1036.9	t=-0.38;
Cord	75	48.8±11.4	2.2-608.6	Prob> | t | =0.7022
Group III				
Maternal	55	41.6±6.5	2.3-211.4	t=1.2;
Cord	56	48.2±9.8	1.4-466.6	Prob> | t | =0.2409
TOTAL				
Maternal	224	45.7±6.0	1.2-1036.9	t=0.29;
Cord	225	46.8±5.5	0.4-631.5	Prob> | t | =0.7722

* Geometric Mean Titre ± Standard Error.

** $p < 0.05$ was denoted a statistically significant difference between maternal and cord sera.

Group I Acellular DTP from birth.
Group II Acellular DTP from 2 months.
Group III Whole-cell DTP from 2 months.

TABLE 10.2 Comparison of pertussis IgG-FHA antibody titres in paired maternal and cord sera (ELISA U/ml).

	N	GMT ± SE*	Range	Paired t-test** (cord-maternal)
Group I				
Maternal	93	82.9±10.2	5.5-618.0	t=0.52;
Cord	94	93.0±17.4	6.2-1376.5	Prob> | t | =0.6048
Group II				
Maternal	74	80.4±10.6	12.7-590.3	t=-1.04;
Cord	73	86.1±10.2	4.9-461.9	Prob> | t | =0.3051
Group III				
Maternal	56	72.2±12.1	12.3-591.0	t=-0.48;
Cord	55	78.5±15.1	5.3-595.4	Prob> | t | =0.6322
TOTAL				
Maternal	223	79.4±6.3	5.5-618.0	t=0.47;
Cord	222	87.1±8.9	4.9-1376.5	Prob> | t | =0.6353

* Geometric Mean Titre ± Standard Error.

** $p < 0.05$ was denoted a statistically significant difference between maternal and cord sera.

Group I Acellular DTP from birth.
Group II Acellular DTP from 2 months.
Group III Whole-cell DTP from 2 months.

TABLE 10.3 Comparison of pertussis IgG-AGG2,3 antibody titres in paired maternal and cord sera in full-term infants (ELISA U/ml).

	N	GMT ± SE*	Range	Paired t-test** (cord-maternal)
Group I				
Maternal	91	173.8±28.8	0.6-1666.3	t=-1.89;
Cord	92	143.7±26.7	0.6-1667.8	Prob> | t | =0.0615
Group II				
Maternal	74	189.8±33.2	0.6-1552.4	t=-0.88;
Cord	74	143.9±27.0	0.6-1656.8	Prob> | t | =0.3826
Group III				
Maternal	55	404.9±203.4	15.7-11228.8	t=-1.52;
Cord	54	522.5±290.5	0.6-13709.0	Prob> | t | =0.1333
TOTAL				
Maternal	220	367.3±82.9	0.6-11228.8	t=0.08;
Cord	220	366.9±113.3	0.6-13709.0	Prob> | t | =0.93

* Geometric Mean Titre ± Standard Error.

** $p < 0.05$ was denoted a statistically significant difference between maternal and cord sera.

Group I Acellular DTP from birth.
Group II Acellular DTP from 2 months.
Group III Whole-cell DTP from 2 months.

TABLE 10.4 Comparison of antibody of full-term infants with low (< 40 units/ml) and high (≥ 40 units/ml) cord anti-PT titres to early and routine acellular and whole-cell pertussis vaccination.

PRE-EXISTING IgG PT TITRE	AGE AT BLOOD SAMPLING TIME (ELISA U/ml) 4 MONTHS	6 MONTHS	9 MONTHS
Group I			
Low titre	100.6 ± 14.3(45)*	156.0 ± 32.4(32)	109.5 ± 17.5(32)
High titre	125.4 ± 39.4(22)	129.1 ± 42.9(21)	155.9 ± 59.0(14)
Group II			
Low titre	84.5 ± 14.2(46)	182.6 ± 24.1(42)	144.1 ± 20.8(36)
High titre	79.2 ± 14.5(19)	241.8 ± 67.5(17)	141.5 ± 28.8(14)
Group III			
Low titre	10.8 ± 2.1(32)	20.9 ± 8.6(32)	13.0 ± 1.9(27)
High titre	43.7 ± 20.9(15)	28.5 ± 16.8(16)	17.2 ± 4.6(12)

* Geometric Mean Titre ± Standard Error (N).

** No statistically significant difference in GMT at 4, 6 or 9 months was noted between low and high titre groups ($p > 0.05$) (unpaired t-test).

Group I Acellular vaccine from birth.
Group II Acellular vaccine from 2 months.
Group III Whole-cell vaccine from 2 months.

TABLE 10.5 Comparison of antibody responses of full-term infants with low (< 40 units/ml) and high (≥ 40 units/ml) cord anti-FHA titres to early and routine acellular and whole-cell pertussis vaccination.

PRE-EXISTING IgG PT TITRE	AGE AT BLOOD SAMPLING TIME (ELISA U/ml)*		
	4 MONTHS	6 MONTHS	9 MONTHS
Group I			
Low titre	60.8 ± 09.3(26))	96.5 ± 26.9(17)	67.9 ± 11.2(16)
High titre	118.4 ± 26.2(41))[a]	179.3 ± 85.5(36)	97.9 ± 17.5(29)
Group II			
Low titre	43.7 ± 8.0(18)	112.6 ± 20.8(18)	130.7 ± 25.6(14)
High titre	52.7 ± 6.0(39)	112.6 ± 19.8(32)	109.0 ± 15.9(28)
Group III			
Low titre	26.8 ± 11.8(18)	49.8 ± 28.3(20)	39.6 ± 14.0(16)
High titre	73.2 ± 26.8(28)	54.3 ± 10.9(31)	60.3 ± 18.1(26)

* Geometric Mean Titre ± Standard Error (N).

a $t = -2.0678$; $p = 0.044$ (unpaired t-test); low titre vs. high titre.

Group I Acellular vaccine from birth.
Group II Acellular vaccine from 2 months.
Group III Whole-cell vaccine from 2 months.

TABLE 10.6 Comparison of pre-existing IgG antibody responses of full-term infants with low (<90 units/ml) and high (≥90 units/ml) pre-existing anti-AGG2,3 titres to early and routine acellular and whole-cell pertussis vaccination.

PRE-EXISTING AGG2,3 TITRE	AGE AT BLOOD SAMPLING TIME (ELISA U/ml)*		
	4 MONTHS	6 MONTHS	9 MONTHS
Group I			
Low titre	99.7 ± 26.4(34)	137.6 ± 45.9(27)	177.4 ± 74.1(21)
High titre	257.5 ± 76.0(31)	308.3 ± 79.8(25)	290.1 ± 67.6(23)
Group II			
Low titre	48.8 ± 08.5(28))	69.9 ± 15.2(29))	149.7 ± 73.0(22)
High titre	128.4 ± 23.2(30))[a]	148.4 ± 25.4(23))[b]	165.1 ± 29.9(22)
Group III			
Low titre	60.5 ± 10.4(18)	409.2 ± 101.0(22)	553.2 ± 127.1(15)
High titre	942.9 ± 431.1(25)	1094.2 ± 338.5(25)	1607.9 ± 441.9(23)

* Geometric Mean Titre ± Standard Error (N).
a p = 0.03;
b P = 0.001 (unpaired t-test); low titre vs. high titre.

Group I Acellular vaccine from birth.
Group II Acellular vaccine from 2 months.
Group III Whole-cell vaccine from 2 months.

TABLE 10.7 Seroconversion* to PT, FHA and AGG2,3 by 9 months of age in full-term infants with low (<40 for PT, FHA; <90 for AGG2,3) and high (≥40 for PT, FHA; ≥90 for AGG2,3) pre-existing IgG antibody titres in recipients of acellular and whole-cell pertussis vaccines.

PRE-EXISTING** IgG ANTIBODY	PERCENTAGE SEROCONVERSION GROUP I	GROUP II	GROUP III
PT			
Low titre	81.0 (38/47)[a]	79 (30/38)[b]	22 (05/23)
High titre	33.4 (08/22)	42 (08/19)	07 (02/27)
FHA			
Low titre	56 (15/27)[c]	85 (39/46)[d]	38 (13/34)[e]
High titre	07 (03/43)	17 (03/18)	00 (00/22)
AGG2,3			
Low titre	35.6 (16/45)[f]	30.9 (13/42)[g]	79.4 (27.34)[h]
High titre	15.2 (05/38)	04.9 (02/41)	40.0 (12.24)

* ≥4-fold rise in titre from pre-vaccination titres.

** The titre of maternally-derived antibodies in Group I was the level in cord blood and that for Groups II and III, pre-vaccination samples taken at 2 months of age.

Group I Acellular vaccine from birth.
Group II Acellular vaccine from 2 months.
Group III Whole-cell vaccine from 2 months.

a-h Indicates significantly greater seroconversion in low titre subgroups - a - p=0.0002; b - p=0.005; c - p=0.000006; d - p=0.000002; e - p=0.0009; f - p=0.019; g - p=0.002; h - p=0.0187.

TABLE 10.8 Maternal and cord pertussis IgG-PT antibody levels in small-for-gestational age (SGA) and appropriate-for-gestational age (AGA) infants (ELISA U/ml).

SAMPLE (n)	GMT ± SE [+]	RANGE	SD*	UNPAIRED T-TEST (UNEQUAL VARIANCE)
Maternal				
SGA (17)	26.0 ± 4.9	1.2-67.5	20.1	t = 2.5661;
AGA (224)	45.7 ± .9	1.2-1036.9	89.0	p = 0.0120**
Cord				
SGA (17)	24.0 ± 5.0	2.0-73.7	20.5	t = 3.0865;
AGA (225)	46.8 ± 5.5	0.4-631.5	82.0	p = 0.0029**
Cord/maternal				
SGA (17)	1.0 ± 0.1	0.26-1.95	0.5	t = 1.4696;
AGA (221)	2.0 ± 0.6	0.01-134.25	9.1	p = 0.1430

[+] Geometric Mean Titre ± Standard Error.
* Standard Deviation.
** P < 0.05 denotes a statistically significant difference.

TABLE 10.9 Maternal and cord pertussis IgG-FHA antibody levels in small-for-gestational age (SGA) and appropriate-for-gestational age (AGA) infants (ELISA U/ml).

SAMPLE (n)	GMT ± SE [+]	RANGE	SD*	UNPAIRED T-TEST (UNEQUAL VARIANCE)
Maternal				
SGA (17)	55.7 ± 10.1	10.7-183.9	41.6	t = 1.9955;
AGA (223)	79.4 ± 6.3	05.5-618.0	93.7	p = 0.0550**
Cord				
SGA (17)	77.1 ± 22.0	4.9-311.0	90.6	t = 0.4218;
AGA (222)	87.1 ± 08.9	4.9-1376.5	132.3	p = 0.6774**
Cord/maternal				
SGA (17)	1.3 ± 0.2	0.28-4.86	1.0	t = 0.1617;
AGA (218)	1.3 ± 0.1	0.08-16.91	1.5	p = 0.8730

[+] Geometric Mean Titre ± Standard Error.
* Standard Deviation.
** P < 0.05 denotes a statistically significant difference.

TABLE 10.10 Maternal and cord pertussis IgG-AGG2,3 antibody levels in small-for-gestational age (SGA) and appropriate-for-gestational age (AGA) infants (ELISA U/ml).

SAMPLE (n)	GMT ± SE +	RANGE	SD*	UNPAIRED T-TEST (UNEQUAL VARIANCE)
Maternal				
SGA (17)	156.2 ± 46.3	25.4-759.4	190.9	t = 2.2235;
AGA (220)	367.3 ± 82.9	09.9-17404.6	1229.2	p = 0.0276**
Cord				
SGA (17)	97.9 ± 27.6	12.1-502.9	113.9	t = 0.2.3067;
AGA (220)	366.9 ± 133.3	0.9-21249.3	1680.1	p = 0.0219**
Cord/maternal				
SGA (17)	0.88 ± 0.14	0.27-2.26	0.59	6 = 1.2437
AGA (216)	1.09 ± 0.10	0.02-15.50	1.40	p = 0.2225

\+ Geometric Mean Titre ± Standard Error.
* Standard Deviation.
** P < 0.05 denotes a statistically significant difference.

TABLE 10.11 Maternal and cord IgG-PT levels in pre-term and full-term infants (ELISA U/ml).

GESTATIONAL AGE	N	GMT ± SE*	RANGE
28-32 weeks			
Maternal	10	58.5 ± 20.8	0.9-157
Cord	9	56.5 ± 29.7	0.9-215
Cord/maternal	5	1.6 ± 0.7	0.1-4.4
33-37 weeks			
Maternal	10	54.8 ± 014.5	
Cord	6	107.5 ± 049.0	02.6-857
Cord/maternal	5	0.66 ± 00.2	12.7-330
39-40 weeks			
Maternal	224	45.7 ± 06.0	1.2-1036.9
Cord	225	46.8 ± 05.5	0.4-631.5
Cord/maternal	221	2.0 ± 0.6	0.01-134.25

* Geometric Mean Titre ± Standard Error.

TABLE 10.12 Maternal and cord IgG-FHA levels in pre-term and full-term infants (ELISA U/ml).

GESTATIONAL AGE	N	GMT ± SE*	RANGE
28-32 weeks			
Maternal	10	97.1 ± 024.9	0.9-211.0
Cord	9	153.9 ± 034.8	17.3-372.0
Cord/maternal	5	21. ± 00.9	0.3-5.6
33-37 weeks			
Maternal	11	127.9 ± 031.2	13.1-324.0
Cord	5	123.1 ± 35.3	13.6-235.0
Cord/maternal	5	1.2 ± 0.3	0.3-1.9
39-40 weeks			
Maternal	223	79.4 ± 6.3	5.5-618.0
Cord	222	87.1 ± 8.9	4.9-1376.5
Cord/maternal	218	1.3 ± 0.1	0.1-16.9

* Geometric Mean Titre ± Standard Error.

TABLE 10.13 Maternal and cord IgG-AGG2,3 levels in pre-term and full-term infants (ELISA U/ml).

GESTATIONAL AGE	N	GMT ± SE*	RANGE
28-32 weeks			
Maternal	10	276.9 ± 150.6	0.9-1287
Cord	9	527.5 ± 219.8	2.4-2027
Cord/maternal		1.9 ± 0.7	0.3-3.9
33-37 weeks			
Maternal	11	266.2 ± 71.9	4.1-689
Cord	5	163.2 ± 43.9**	8.1-247
Cord/maternal	5	0.8 ± 0.2	0.5-1.4
39-40 weeks			
Maternal	220	367.3 ± 82.9	0.6-11228.8
Cord	220	366.9 ± 113.3	0.6-13709.0
Cord/maternal	216	1.0 ± 0.1	0.02-15.5

* Geometric Mean Titre ± Standard Error.

** Cord GMT was significantly lower than the maternal GMT ($p = 0.0485$).

TABLE 10.14 Maternal and cord IgG-diphtheria antibody levels in pre-term and full-term infants (ELISA U/ml).

GESTATIONAL AGE	N	GMT ± SE*	RANGE
28-32 weeks			
Maternal	10	0.5±0.2	0.01-2.1
Cord	9	0.5±0.3	0.03-2.7
Cord/maternal	5	6.9±5.3	0.1-28.0
33-37 weeks			
Maternal	11	0.4±0.1	0.06-1.1
Cord	5	0.3±0.1	0.06-0.8
Cord/maternal	5	1.0±0.4	0.2-2.0
Full term			
Maternal	34	0.3±0.07	0.01-1.72
Cord	41	0.4±0.10	0.01-2.68
Cord/maternal	28	6.2±3.24	0.04-72.0

* Geometric Mean Titre ± Standard Error.

TABLE 10.15 Maternal and cord IgG-tetanus antibody levels in pre-term and full-term infants (ELISA U/ml).

GESTATIONAL AGE	N	GMT ± SE*	RANGE
28-32 weeks			
Maternal	10	0.3±0.1	0.01-1.1
Cord	9	0.3±0.2	0.02-1.8
Cord/maternal	5	3.5±2.8	0.05-12.0
33-37 weeks			
Maternal	11	0.3±0.1	0.03-0.6
Cord	5	0.2±0.1	0.03-0.4
Cord/maternal	5	0.5±0.2	0.1-1.1
full-term			
Maternal	34	0.2±0.09	0.01-0.8
Cord	41	0.4±0.05	0.01-1.3
Cord/maternal	28	3.1±1.6	0.02-32.0

* Geometric Mean Titre ± Standard Error.

TABLE 10.16 Maternal and cord IgG-PT levels in infants with subclinical *B. pertussis* infection.

	N	GMT ± SE	RANGE	PROB > | t | *
Maternal				
Cases	10	29.7 ± 6.6	14.8-84.6	0.081
Non-cases	224	45.7 ± 5.9	1.2-1036.9	
Cord				
Cases	10	33.5 ± 8.1	7.8-79.4	0.190
Non-cases	225	46.8 ± 5.5	0.4-631.5	
Cord/maternal				
Cases	10	1.2 ± 0.3	0.3-3.2	0.267
Non-cases	221	1.9 ± 0.6	0.01-134.2	

+ Geometric Mean Titre ± Standard Error.
* t-test (unequal variance);

$p < 0.05$ denotes a statistically significant difference between levels in cases and non-cases.

TABLE 10.17 Maternal and cord IgG-FHA levels in infants with subclinical *B. pertussis* infection.

	N	GMT ± SE	RANGE	PROB> | t | *
Maternal				
Cases	10	170.2 ± 80.9	16.4-711.1	0.292
Non-cases	223	79.4 ± 6.3	5.5-618.0	
Cord				
Cases	10	119.6 ± 53.6	16.2-461.9	0.565
Non-cases	223	87.1 ± 8.9	4.9-1376.5	
Cord/maternal				
Cases	10	1.1 ± 0.4	0.09-4.2	0.579
Non-cases	218	1.3 ± 0.1	0.08-16.9	

\+ Geometric Mean Titre ± Standard Error.
* t-test (unequal variance);

$p < 0.05$ denotes a statistically significant difference between levels in cases and non-cases.

TABLE 10.18 Maternal and cord IgG-AGG2,3 levels in infants with subclinical *B. pertussis* infection.

	N	GMT ± SE	RANGE	PROB> | t | *
Maternal				
Cases	10	362.3 ± 169.2	19.6-1630.0	0.979
Non-cases	220	367.3 ± 82.9	0.9-17404.6	
Cord				
Cases	10	134.7 ± 29.6	27.7 ± 290.3	0.049
Non-cases	220	366.9 ± 113.3	0.9-21249.3	
Cord/maternal				
Cases	10	1.0 ± 0.2	0.05-1.7	0.724
Non-cases	216	1.1 ± 0.1	0.02-15.5	

+ Geometric Mean Titre ± Standard Error.
* t-test (unequal variance);

$p < 0.05$ denotes a statistically significant difference between levels in cases and non-cases.

TABLE 10.19 Pertussis infection in pre-term infants: patient details.

CASE	GESTATIONAL AGE (weeks)	AGE AT WHICH INFECTION OCCURRED (months)	NO. OF DOSES OF DTP ADMINISTERED AT TIME OF INFECTION
1	28	$>2\leq4$	1
2	34	$>2\leq4$	2
3	34	$>4\leq6$	1
4	35	$>6\leq9$	3

TABLE 10.20 Geometric mean titres (GMTs) of IgG antibodies to PT, FHA and AGG2,3 following whole-cell vaccination of pre-term infants.

GROUP SAMPLING TIME	GMT ± SE* IgG-PT	 IgG-FHA	 IgG-AGG2,3
28-32 weeks			
Pre-vaccination	56.5 ± 29.7(9)	153.9 ± 34.8(9)	527.5 ± 219.8(9)
2 months	20.9 ± 13.8(6)	26.2 ± 10.2(6)	42.5 ± 14.6(6)
4 months	30.8 ± 12.8(5)	93.4 ± 38.9(4)	227.5 ± 146.3(4)
6 months	27.5 ± 26.6(2)	---	---
9 months	16.1 ± 12.0(3)	---	---
33-37 weeks			
Pre-vaccination	76.9 ± 26.3(15)	123.1 ± 35.3(5)	163.2 ± 43.9(5)**
2 months	18.3 ± 6.5(13)	72.4 ± 36.9(7)	174.9 ± 86.2(7)
4 months	20.6 ± 8.1(9)	3.8 ± 2.9(2)	31.3 ± 11.8(2)
6 months	---	---	659.3 ± 616.8(3)

* Geometric mean ± Standard Error.

** 2-month GMT was significantly lower than the pre-vaccination titre ($p = 0.0279$).

TABLE 10.21 Geometric mean titre (GMTs) of IgG antibodies to diphtheria and tetanus toxins following whole-cell pertussis vaccination of pre-term infants (ELISA U/ml).

GROUP SAMPLING TIME	n	GMT ± SE* DIPHTHERIA	 TETANUS
28-32 weeks			
Pre-vaccination	9	0.5±0.3	0.3±0.1
2 months	6	0.3±0.1	0.18±0.03
4 months	5	0.3±0.1	0.28±0.04
6 months	2	1.0±0.9	0.36±0.04
9 months	3	5.5±5.4	0.33±0.05
33-37 weeks			
Pre-vaccination	11	0.4±0.1	0.30±0.10
2 months	5	0.3±0.1	0.14±0.03
4 months	7	0.2±0.1	0.31±0.24
6 months	3	1.2±0.9	0.51±0.06
9 months	5	1.2±0.7	0.38±0.05
Full-term			
Pre-vaccination	41	0.4±0.1	0.37±0.06
2 months	13	0.1±0.02	0.27±0.04
4 months	10	0.3±0.2	0.35±0.14

* Geometric mean ± Standard Error.

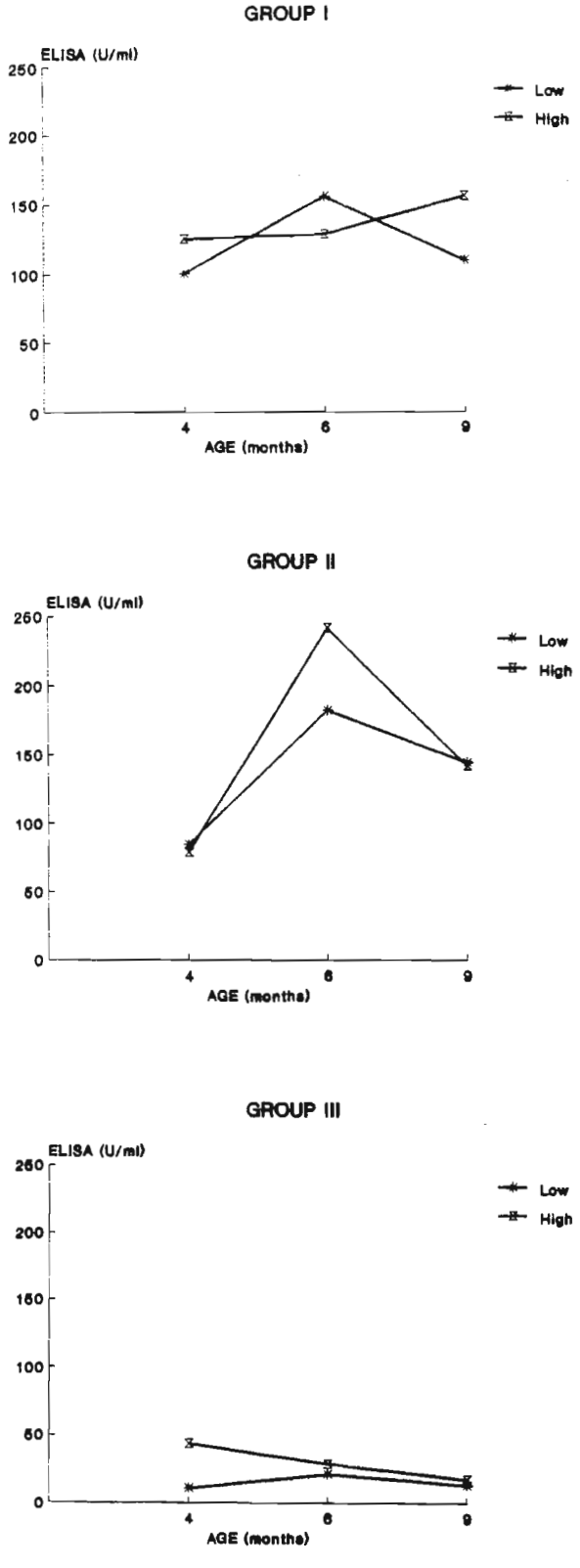


FIGURE 10.1 Comparison of antibody responses of infants with low (<40 U/ml) and high (≥40 U/ml) pre-existing anti-PT titres to early and routine acellular and whole-cell pertussis vaccination.

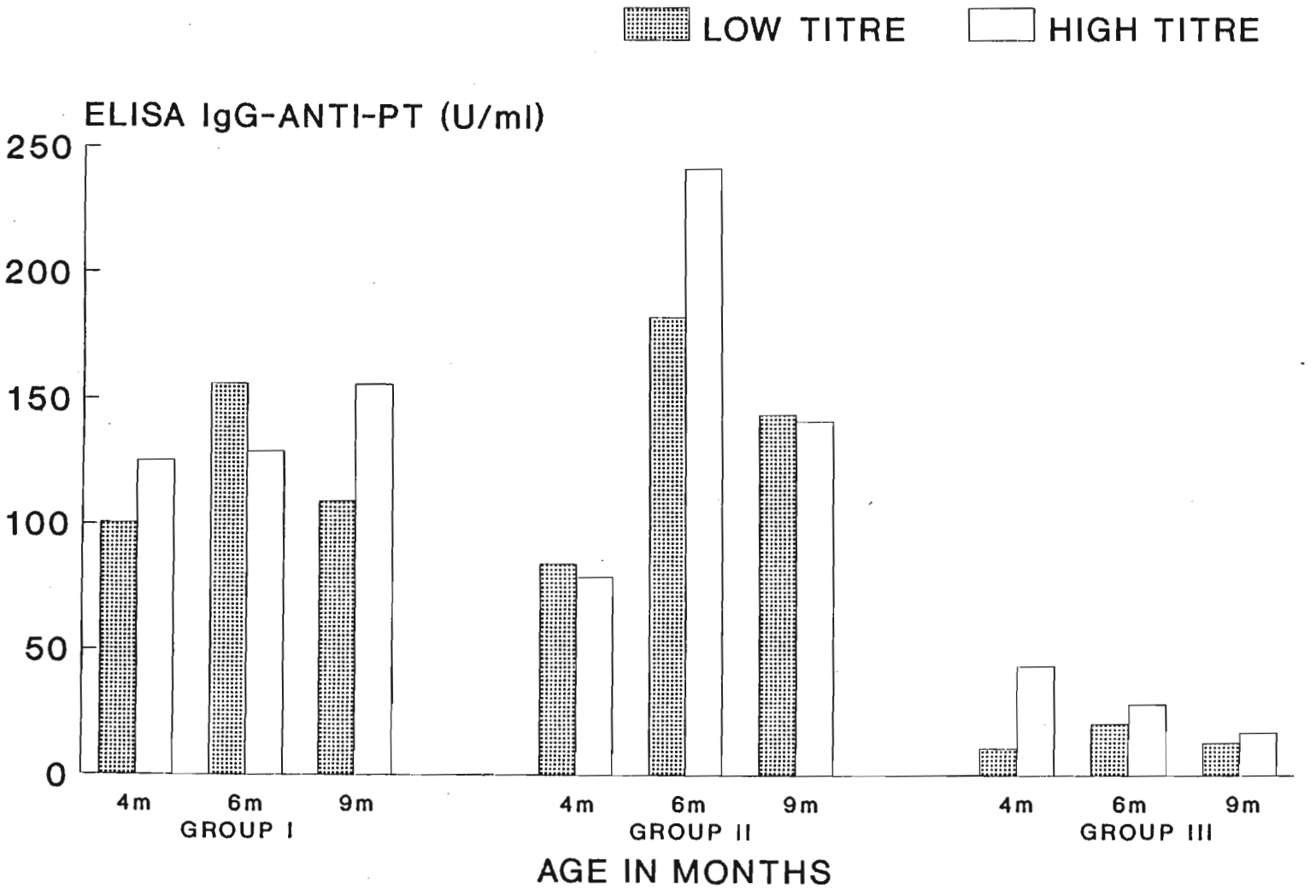


FIGURE 10.2 Relationship of IgG-PT titres to age in infants with high (≥ 40 U/ml) and low (< 40 U/ml) pre-existing titres receiving acellular and whole-cell pertussis vaccine.

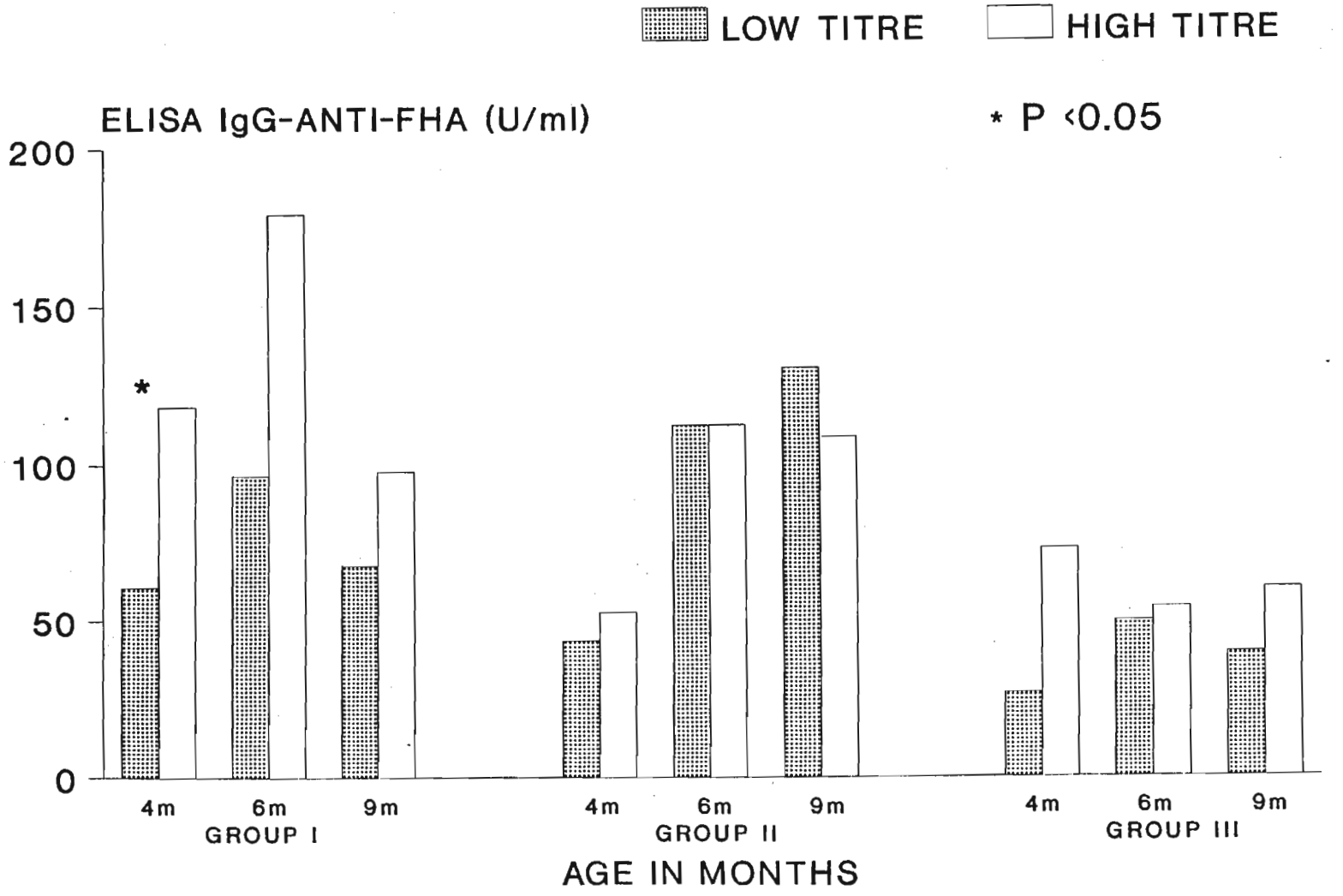


FIGURE 10.3 Relationship of IgG-FHA to age in infants with high (≥40 U/ml) and low (<40 U/ml) pre-existing titres receiving acellular or whole-cell pertussis vaccine.

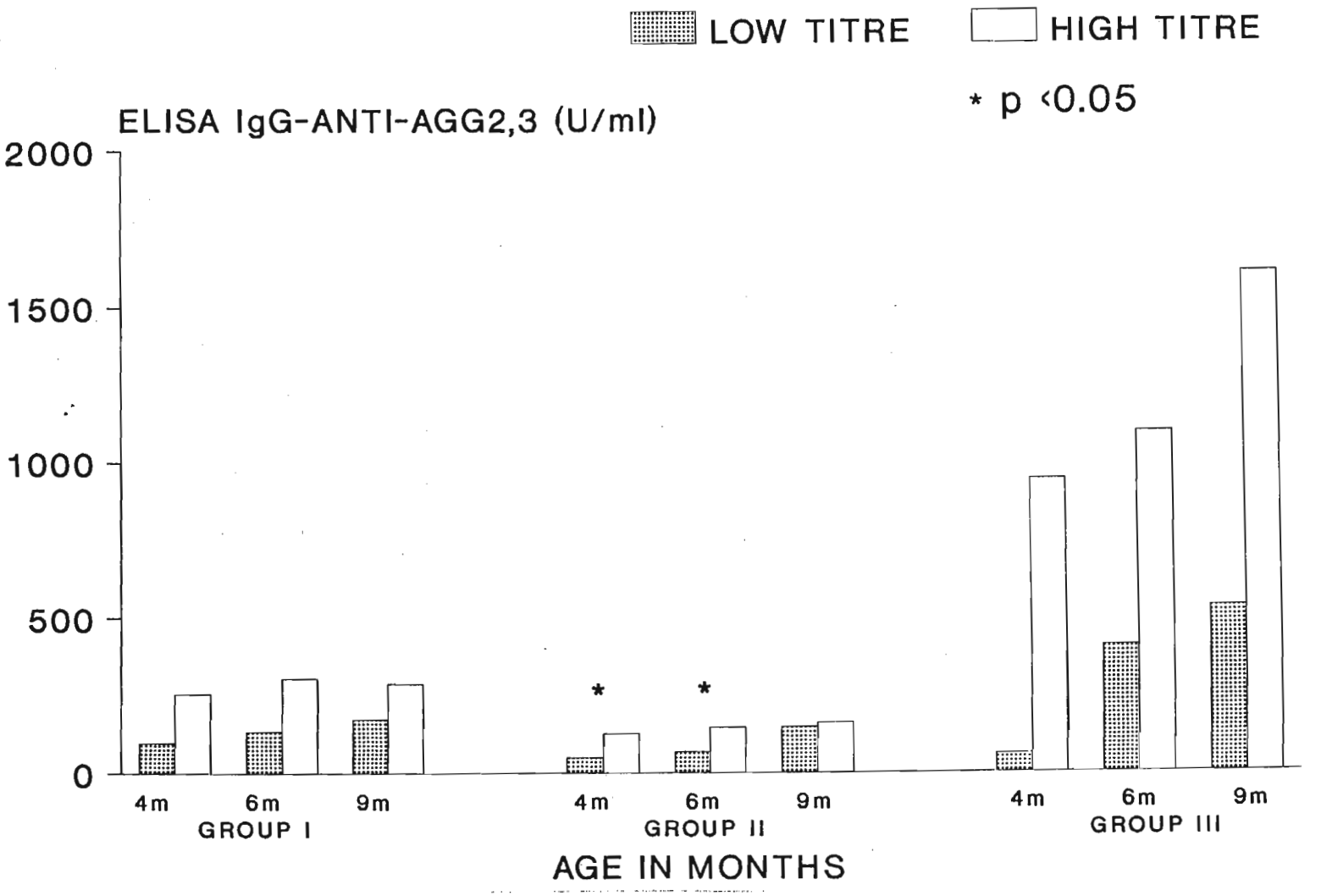


FIGURE 10.4 Relationship of IgG-AGG2,3 titres to age in infants with high (≥90 U/ml) and low (<90 U/ml) pre-existing titres receiving acellular or whole-cell pertussis vaccine.

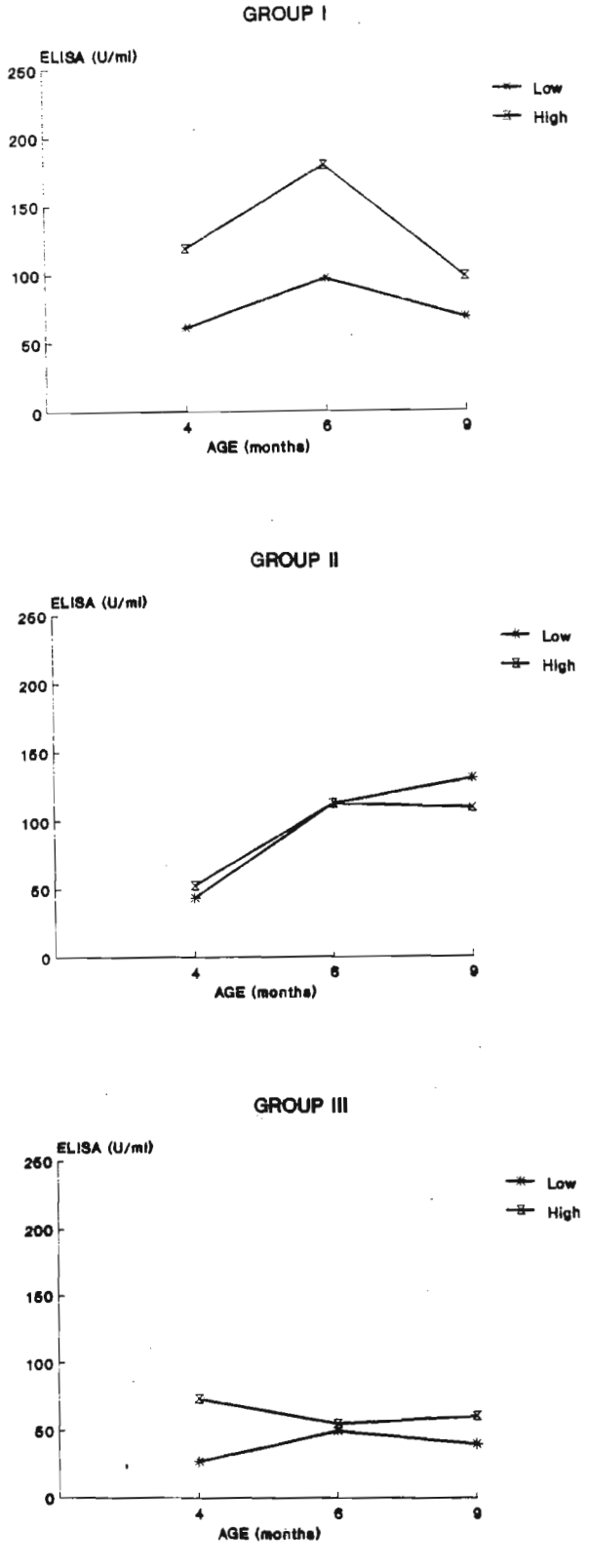


FIGURE 10.5 Comparison of antibody responses of infants with low (< 40 U/ml) and high (⩾40 U/ml) pre-existing anti-FHA titres to early and routine acellular and whole-cell pertussis vaccination.

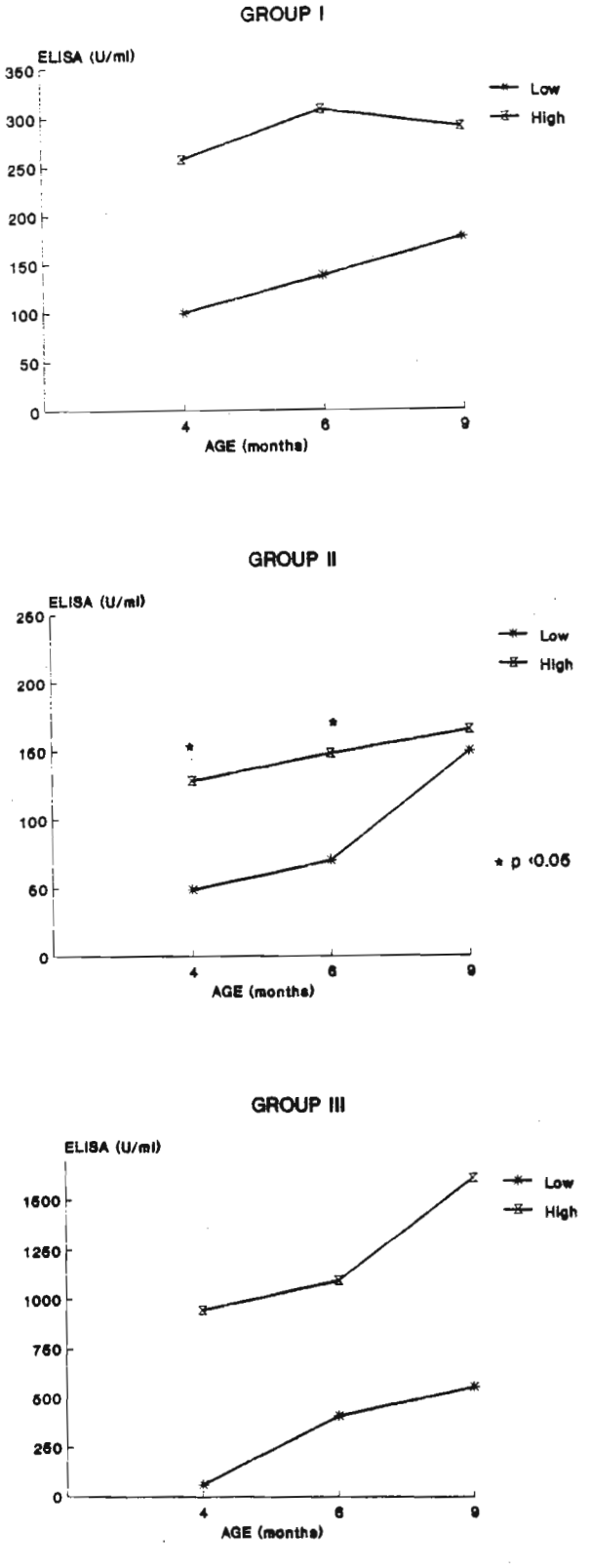


FIGURE 10.6 Comparison of antibody responses of infants with low (<90 U/ml) and high (≥90 U/ml) pre-existing anti-AGG2,3 titres to early and routine acellular and whole-cell pertussis vaccination.

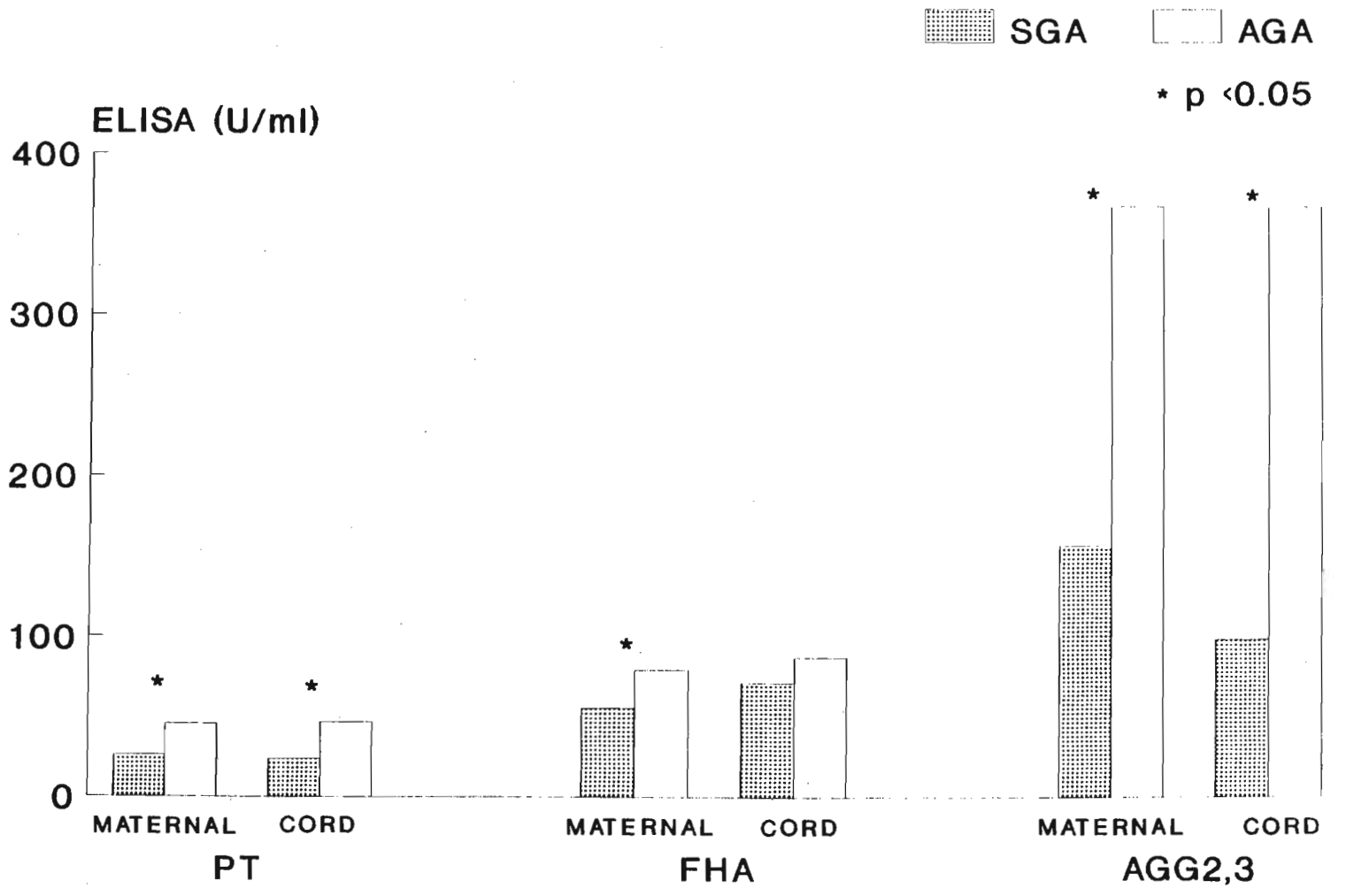


FIGURE 10.7 Maternal and cord pertussis IgG antibody levels in small-for-gestational age (SGA) and appropriate-for-gestational-age (AGA) infants.

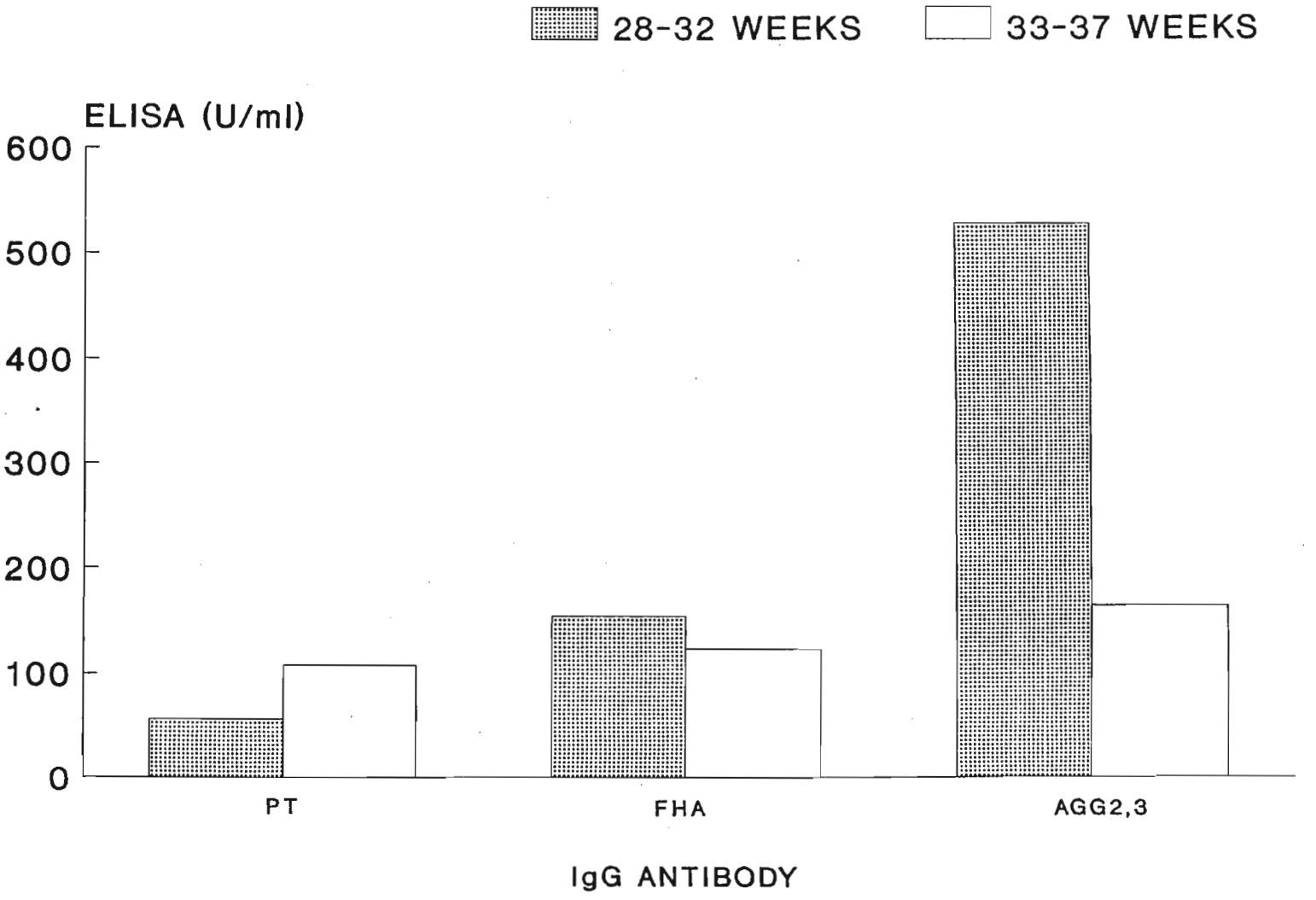


FIGURE 10.8 Pertussis IgG antibody levels in cord blood of pre-term infants.

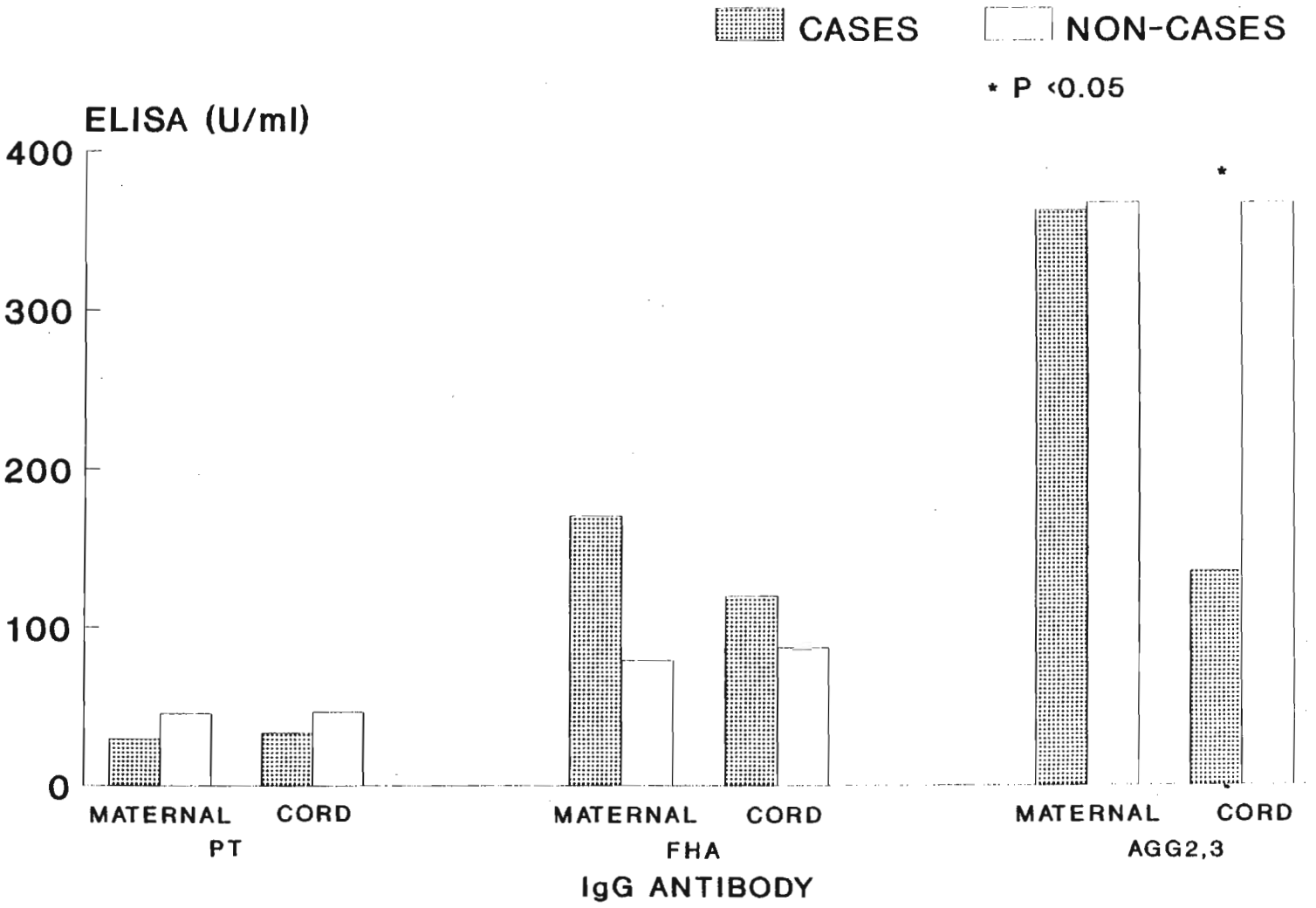


FIGURE 10.9 Maternal and cord pertussis IgG antibody levels in full-term infants with subclinical pertussis infection.

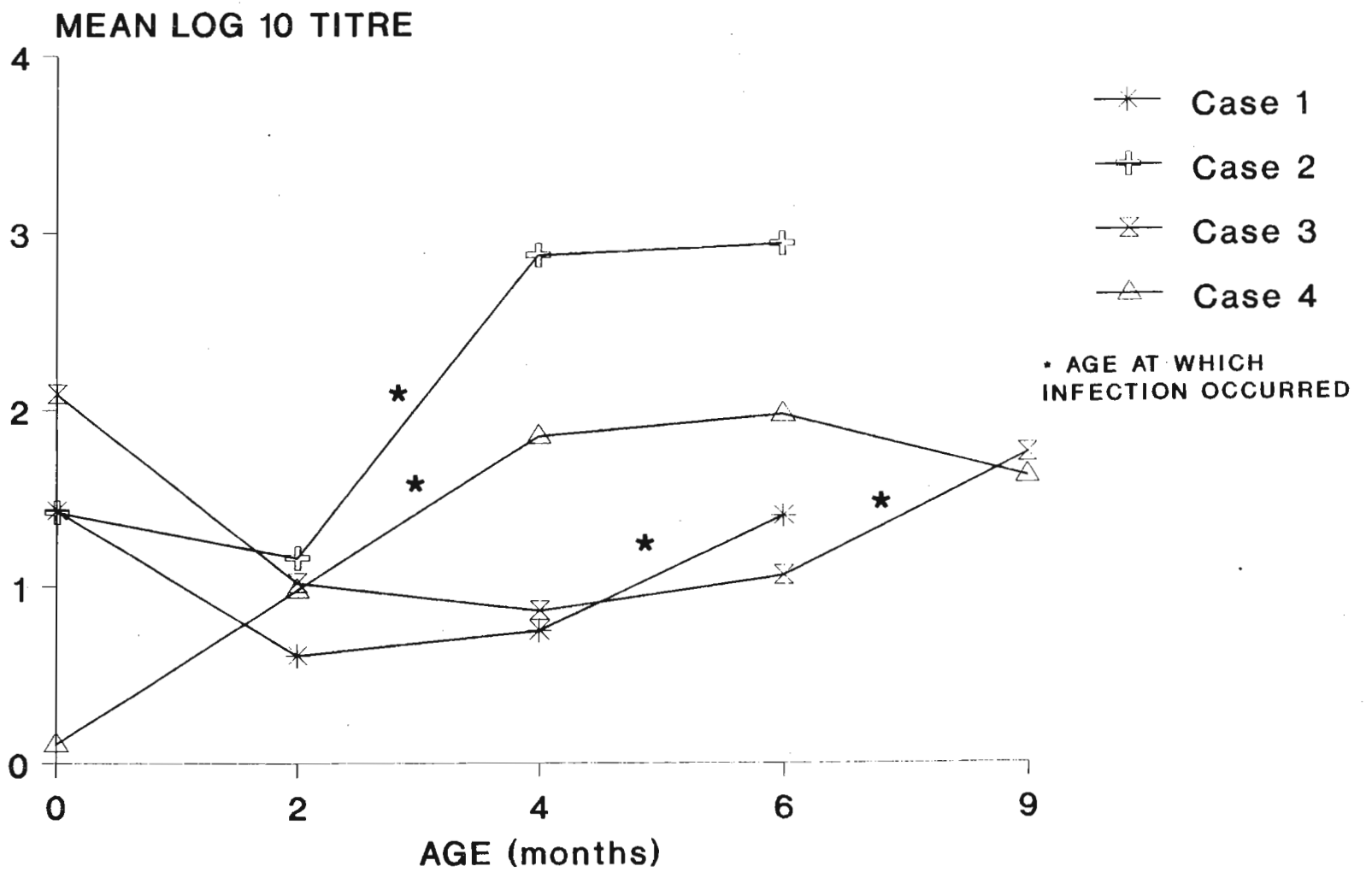


FIGURE 10.10 IgG-PT antibody profiles in 4 pre-term infants who developed subclinical pertussis by 9 months of age.

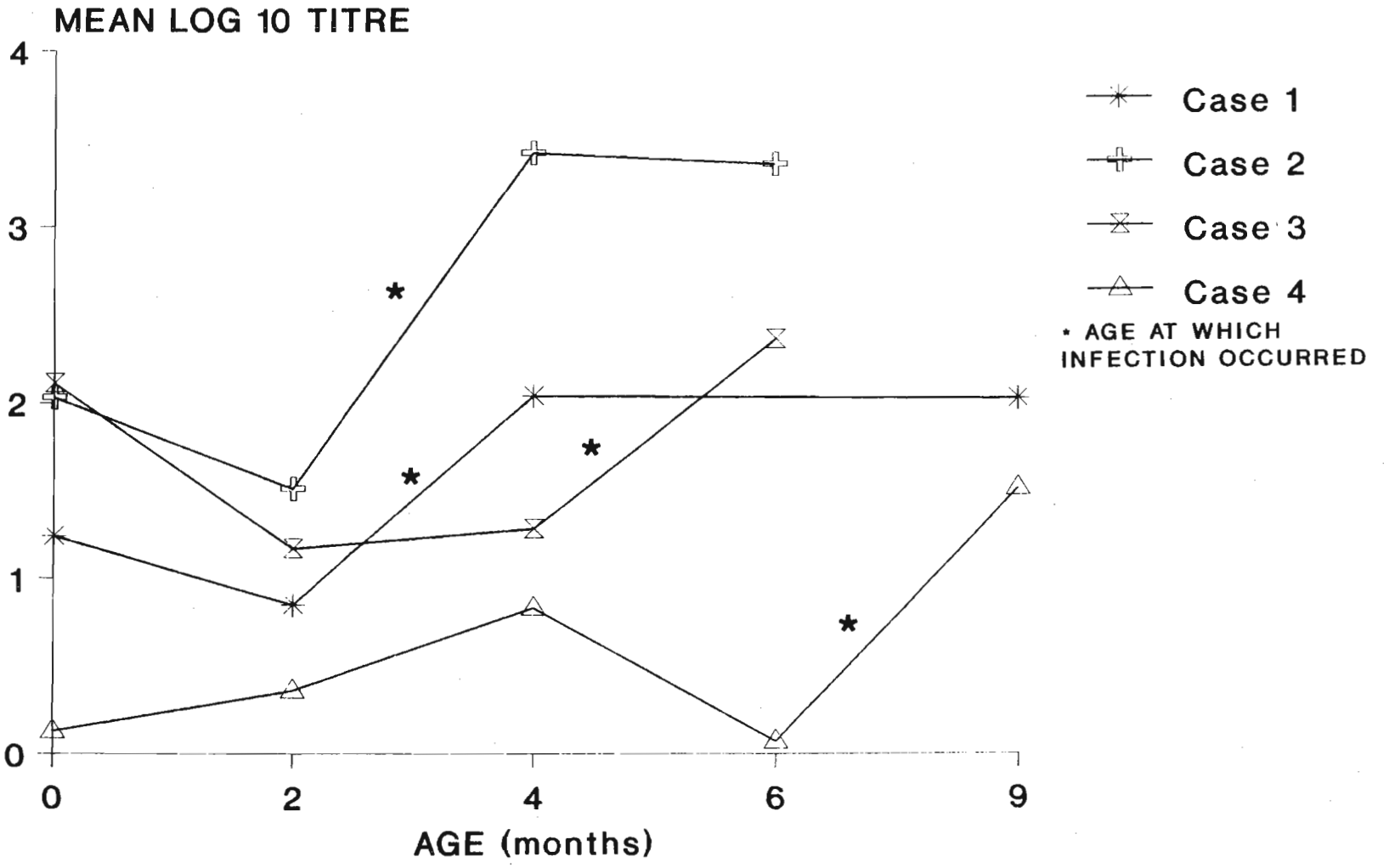


FIGURE 10.11 IgG-FHA antibody profiles in pre-term infants who developed subclinical pertussis by 9 months of age.

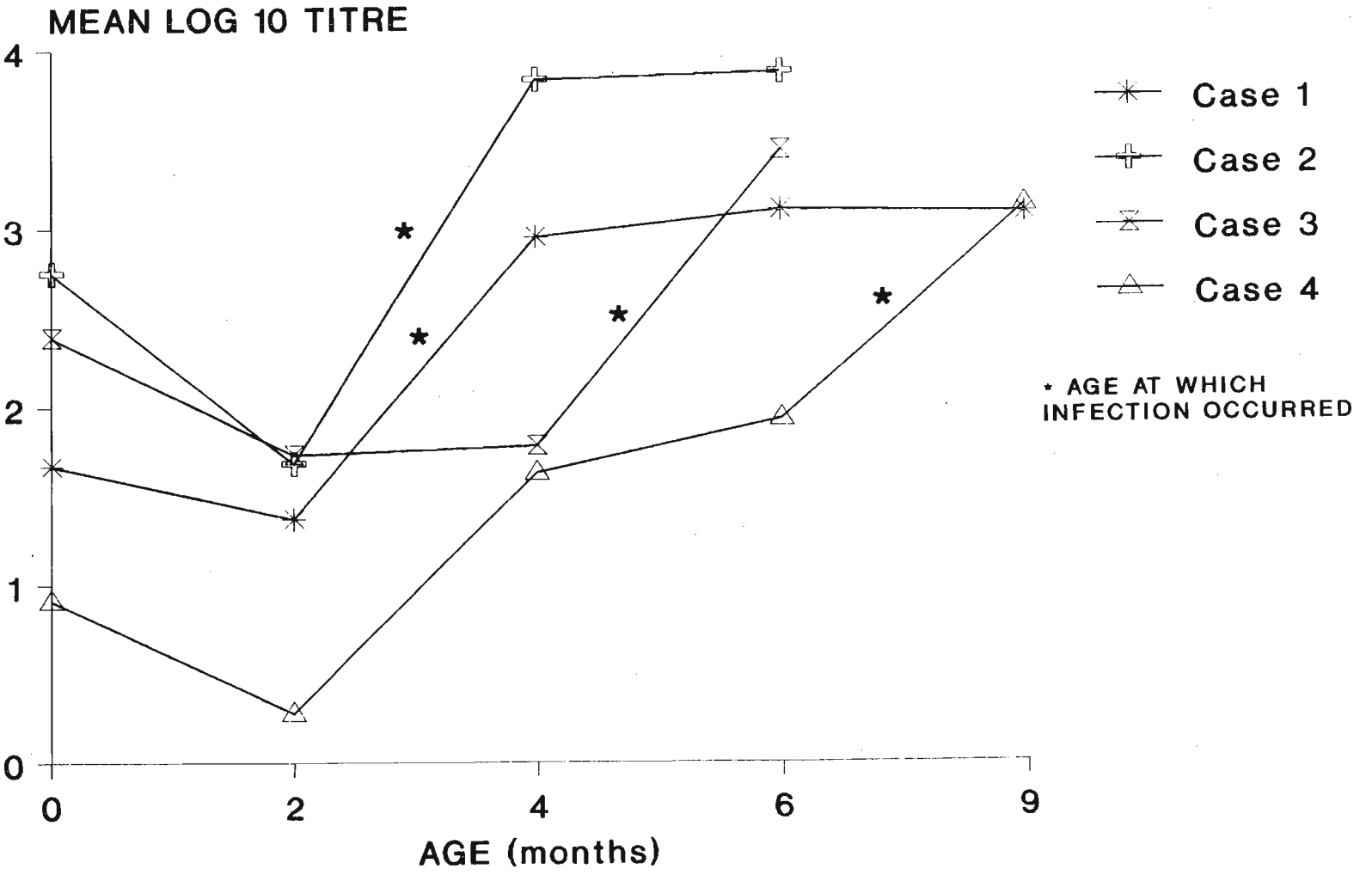


FIGURE 10.12 IgG-AGG2,3 antibody profiles in pre-term infants who developed subclinical pertussis by 9 months of age.

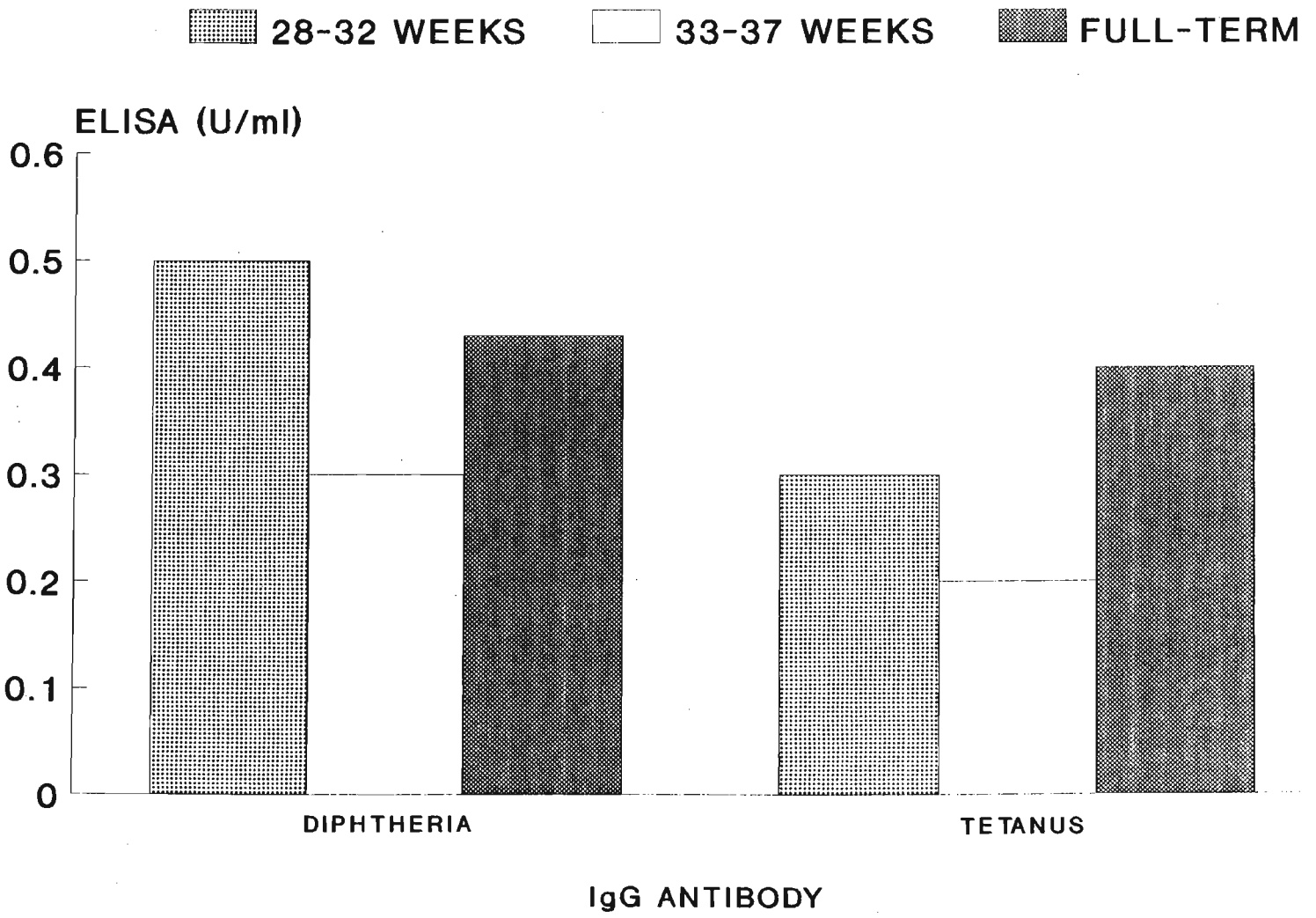


FIGURE 10.13 Diphtheria and tetanus IgG antibody levels in cord blood of pre-term and full-term infants.

CHAPTER 11

EFFECT OF PROTEIN-ENERGY MALNUTRITION ON SAFETY AND IMMUNOGENICITY OF ACELLULAR AND WHOLE-CELL PERTUSSIS VACCINES

11.1 OBJECTIVES

Major objective

To evaluate the effect of protein-energy malnutrition (PEM) on the immunogenicity and reactogenicity of acellular and whole-cell pertussis vaccines.

Subsidiary objectives

To evaluate the serologic responses and post-vaccination events after acellular and whole-cell pertussis vaccines in the following groups of infants:

i. Small-for-gestational-age (SGA) infants who were well-nourished by 2 months of age.

ii. SGA infants who developed PEM before completion of the primary vaccination course, ie. by 9 months of age.

iii. Appropriate-for-gestational-age (AGA) infants who developed PEM before completion of the primary vaccination course, ie. by 9 months of age.

11.2 SUMMARY

During analysis of the data from a phase II study of the immunogenicity and safety of acellular pertussis vaccines (Chapter 8), 25 infants with protein-energy-malnutrition (PEM) were detected on the basis of anthropometric indices. Seventeen infants were small-for-gestational age (SGA) of whom 9 developed PEM by the age of 9 months. Eight other infants developed post-natal PEM before the completion of the primary vaccination course.

Vaccine groups

Infants were vaccinated at 2, 4 and 6 months of age with either acellular pertussis-diphtheria-tetanus (A-DTP) (Groups I and II), or whole-cell pertussis-diphtheria-tetanus (W-DTP) (Group III). In addition, at birth, Group I received an additional dose of A-DTP and Group II a saline placebo.

Post-vaccination events were recorded for 14 days after each dose. Paired sera were collected from mother-infant pairs at birth, before vaccination at 2, 4 and 6 months, and at 9 months of age. Serum IgG antibodies to pertussis toxin (PT), filamentous haemagglutinin (FHA), and agglutinogens 2,3 (AGG2,3) were assayed by ELISA.

The following indices were evaluated in malnourished infants; (i) anthropometric indices of nutritional status, (ii) intercurrent illnesses including pertussis infection, (iii) local and systemic post-vaccination events, (iv) transplacental transfer of pertussis antibodies and (v) serologic responses to vaccination. Results were compared with those obtained in well-nourished age and vaccine-matched cohorts.

Overall, peak titres and seroconversion rates of all three antibodies were not significantly different in malnourished infants though final anti-AGG2,3 titres (at 9 months of age) in Group III were significantly lower ($p = 0.035$).

Although peak and final PT and FHA antibody titres in many SGA and malnourished infants were lower than the geometric mean titre (GMT) in well-nourished infants and peak response were attained at a later age, malnutrition did not significantly affect the response to acellular DTP. Peak and final AGG2,3 antibody titres were similar in SGA, malnourished and well-nourished infants.

Maternal and cord IgG-PT and AGG2,3 titres were significantly lower ($p < 0.05$) and maternal IgG-FHA marginally lower ($p \approx 0.05$) in SGA infants compared to cohorts although the placental transfer was equally efficient in both groups.

Incidence of minor local and systemic post-vaccination symptoms was not significantly different in PEM and well-nourished groups; although induration at injection site and irritability

were more frequently reported in the latter. No major neurologic post-vaccination symptoms to either vaccine were reported in SGA infants or infants with PEM at the time of vaccination. No significant difference was noted in the incidence of major symptoms in PEM (either SGA or post-natal) as compared with well-nourished infants.

One male infant (Group I) who was malnourished at birth and who had been given two doses of A-DTP developed clinical signs of pertussis infection between 2 and 4 months of age. Pertussis antibody levels immediately prior to infection were not significantly different from those of uninfected age-matched cohorts. The percentage of infants afflicted with common childhood illnesses were similar in PEM (either SGA or post-natal) and well-nourished groups (46 vs. 43.2%) although the former group incurred significantly more illnesses at an earlier age (≤ 6 months) ($p = 0.05$, chi square).

These findings, albeit preliminary given the small numbers of subjects studied, suggest that acellular pertussis vaccine may be incorporated into routine vaccination schedules followed in developing countries with the expectation that adequate antibody responses will be provoked in SGA infants and in infants who develop post-natal PEM and that the incidence of vaccine-related adverse effects will be no higher than in well-nourished infants.

11.3 INTRODUCTION

There is considerable evidence that protein-energy malnutrition (PEM) during childhood leads to a decrease in cell-mediated immunity (CMI) and impairs the host reaction to infections. This results from morphological changes in the immune system characterised by involution of the thymus and a decreased number of lymphocytes in the lymphoid organs (Chandra, 1979). Malnourished children may therefore have an impaired immune response to vaccines which involve CMI such as BCG and smallpox.

Most research has shown no apparent modification of humoral immunity in PEM though data on

specific serum antibodies are conflicting (Alvarado & Luthringer, 1971; Keet and Thom, 1969; Najjar *et al.*, 1969, El-Gholm *et al.*, 1970). Serum antibody responses to vaccination in PEM seem to vary according to the type and form of antigen used (Pretorius & De Villiers, 1962; Brown & Katz, 1965).

Impaired antibody response has been reported to yellow-fever vaccine (Brown & Katz, 1966), killed influenza A Hong Kong virus (Jose *et al.*, 1970) and tobacco mosaic virus (Gell, 1948). Antibody formation after administration of typhoid vaccine is variously reported as normal or inadequate.

With the exception of the above, vaccination research has shown no apparent decrease or delay of antibody synthesis against most bacterial and viral vaccines in malnourished children (Ajjan, 1989). Normal serum antibody responses, comparable to those in well-nourished infants have been demonstrated to pneumococcal polysaccharide (Work *et al.*, 1973), diphtheria and tetanus toxoids (Chandra, 1972; Ghosh *et al.*, 1980), and polio, smallpox and measles vaccine (Brown & Katz, 1965) in moderately malnourished infants.

Limited data are available concerning the use of other Expanded Programme on Immunisation (EPI) vaccines in PEM. The use of measles, BCG, trivalent oral polio vaccine (TOPV) and conventional whole-cell DTP in moderate malnutrition appears to be safe. The World Health Organisation (WHO) does not consider malnutrition to be a contraindication to vaccination (Galazka *et al.*, 1984).

Acellular pertussis vaccines are currently being evaluated in a number of collaborative trials administered by the WHO. Early evidence suggests that, at a minimum, these vaccines are a safer and equally effective alternative to currently used whole-cell vaccines which have variable efficacy and may be associated with a wide range of adverse effects (Sato *et al.*, 1984; Edwards *et al.*, 1986)

Mild to moderate PEM is the most common prevailing type of nutritional disorder in the Third World. Morbidity and mortality from pertussis is higher in these countries and a large proportion of infants infected with pertussis will therefore be malnourished. Nutritional status deteriorates after onset of the disease and contributes to the higher mortality from pertussis in developing countries (Christie, 1987). In 1988 the estimated annual number of deaths from neonatal pertussis in developing countries was 560,000 (WHO, 1988). In underprivileged communities a high proportion of infants weigh <2,500 at birth and many develop post-natal PEM, hence the number of infants that are malnourished at and during the time of routine primary vaccination is high. Nutrition, immunity and infection are intricately linked to one another. Nutritional deficiencies influence various components of the immune system. The extent and severity of immunological impairment depends on the presence of infection and on the age of onset of nutritional deprivation, among other factors. In developed countries immune function has been shown to be compromised in SGA infants.

The need for defining the nature and extent of immunological impairment, if any, to acellular pertussis vaccine in SGA infants and in PEM is therefore of major importance. The principal objectives of this investigation were therefore to evaluate the effect of intra-uterine malnutrition on the subsequent response to acellular vaccine and to conventional whole-cell DTP; and to determine whether the serologic response to either vaccine was compromised in infants who developed PEM before the completion of the primary vaccination course.

11.4 SUBJECTS AND METHODS

11.4.1 STUDY DESIGN

The present chapter deals with a subset of a study sample of an open unblinded immunogenicity and safety trial of acellular and whole-cell pertussis vaccines in neonates and very young African infants. This subset included all infants who were small-for-gestational age

(SGA) (N = 17) or who developed PEM (N = 8) during the study period, ie. from birth to 9 months of age.

In the immunogenicity and safety study, healthy full-term infants were enrolled at birth and assigned in sequence at birth to one of three vaccine groups of 115 infants each for receipt of either acellular (Groups I and II) or whole-cell (Group III) DTP according to routine vaccination schedules at 2, 4 and 6 months of age. In addition, at birth, Group I received a neonatal dose of A-DTP and Group II a saline placebo injection. Mothers and nursing staff were unaware of which vaccine was administered. In addition to the above, in accordance with WHO recommendations, all infants received BCG and TOPV at birth; and measles vaccine at 9 months of age. Sera for antibody assays were collected from mothers' blood and cord blood at birth and immediately before each vaccination, and were coded and frozen at -20°C until analysis.

The following indices were evaluated for all infants at birth and at 2, 4, 6 and 9 months of age -

i. Anthropometric measurements of nutritional status.

ii. Pertussis infection: (a) well recognised clinical signs, or (b) serologic evidence, ie. a rapid and marked (≥ 4-fold) rise in AGG2,3 and either or both PT and FHA antibodies between 2 consecutive sera at the time of postulated infection followed by a rapid decline in levels.

iii. Intercurrent illnesses.

iv. Local and systemic post-vaccination events.

v. Transplacental transfer of pertussis antibodies.

vi. Serologic responses to vaccination.

Immunogenicity and safety of pertussis vaccination in PEM (SGA or post-natal PEM) were compared with that in 317 age and vaccine-matched well-nourished cohorts. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Natal and written informed consent was obtained from all parents.

11.4.2 ASSESSMENT OF NUTRITIONAL STATUS

The World Health Organisation defines protein-energy-malnutrition as "a range of pathological conditions arising from coincident lack in varying proportions of proteins and calories".

Nutritional status of subjects was assessed at each clinic visit by anthropometric indices of length- and weight-for-age; and clinical features of PEM, vitamin or trace-element deficiencies. Weight was measured on a beam balance scale with the child unclothed, to the nearest 10g. Length was measured using a firm horizontal board with a fixed headpiece and a sliding footpiece. The distance between the headpiece and footpiece was recorded to the nearest 0.1 cm.

The National Centre for Health Statistics (NCHS, 1976) Reference Population was used as a standard. The data from this reference population are available for both sexes as percentile curves of weight-for-age, height-for-age, head circumference and weight-for-length at selected intervals including the 3rd, 5th, 95th and 97th percentile (Appendix 4,5). These standards have been shown to apply to African children in South Africa (Coovadia *et al.*, 1978).

Data for an individual infant (sex, age, length and weight) were used to place the infant in the appropriate percentile. Infants with a weight-for-age less than the 3rd percentile were considered underweight (WHO Classification System). Infants with a length-for-age less than 90% of the 50th percentile were considered stunted (CDC Classification System). Infants with a weight- and length-for-age <5th percentile were considered to be wasted (Waterlow *et al.*, 1977).

Low birthweight (LBW) was defined as a birth weight less than 2500g. Small-for-gestational-age (SGA) was defined as a birth weight or weight-for-length on or below the 10th percentile curve (Lubchenco *et al.*, 1966).

11.4.3 SUBJECTS

The study sample initially comprised 345 neonates, 17 of whom were SGA. Nutritional status reverted to normal by 2 months of age in all SGA infants, however 9 of the infants developed PEM before the age of 9 months. Eight other infants who were well-nourished at birth developed PEM by 9 months of age. No infant developed clinical features of marasmus, kwashiorkor, vitamin or trace-element deficiencies. Of the total of 25 SGA and PEM infants, 16 were female.

Infants were classified according to nutritional status into the following groups -

i. SN:SGA infants who were well-nourished by 2 months of age.

ii. SP:SGA infants who developed PEM by 9 months of age.

iii. NP:well-nourished (AGA) infants who developed PEM by 9 months of age.

Details of malnourished infants in the 3 vaccine groups are given below -

Group I (N = 5): Three infants were SGA, 2 of whom developed PEM during the study period. Two other infants, well-nourished at birth also developed PEM during the study period. (SN: N = 1, SP: N = 1, NP: N = 2).

Group II (N = 12): Eight infants were SGA, 4 of whom subsequently developed PEM. Four other infants developed PEM during the study period. (SN: N = 4, SP: N = 4, NP: N = 4).

Group III (N = 8): Six infants were SGA, 3 of whom subsequently developed PEM. Two other infants developed PEM during the study period. (SN: N = 3, SP: N = 3, NP: N = 2).

Nutritional indices of all the above infants at various ages are given in Table 11.1

Three-vaccine-unrelated deaths occurred in well-nourished infants (none of whom comprise the subjects in this study) before the age of 2 months. Of the 342 remaining infants, 256 (75%) returned at 2 months of age, 232 (68%) at 4 months, 198 (58%) at 6 months and 175 (51%) at 9 months of age.

11.4.4 VACCINES AND VACCINE ADMINISTRATION

The purified acellular DTP preparation used (J-NIH-6) was supplied by the Japanese National Institute of Health and the Kanonji Institute, Research Foundation for Microbial Diseases (BIKEN) of Osaka University, Japan as a single lot (Lot 21B). The vaccine was aluminium adsorbed and contained co-purified PT and FHA in a 1:1 ratio, 28 LF units diphtheria toxoid and 7 LF units tetanus toxoid per 0.5 ml dose.

The conventional whole-cell DTP used was supplied by the South African Institute for Medical Research (SAIMR), Johannesburg, South Africa as a single lot (Lot A595). The vaccine was also aluminium adsorbed and contained 10×10^9 *B. pertussis*, 25 LF units diphtheria toxoid and 6 LF units tetanus toxoid per 0.5 ml dose.

A-DTP and the saline placebo were administered by the subcutaneous route, and W-DTP by the intramuscular route; all to the left thigh in 0.5 ml doses. Vaccination was postponed if the infant had an acute illness and fever.

11.4.5 EVALUATION OF POST-VACCINATION EVENTS AND CLINICAL FOLLOW-UP (ESPECIALLY INTERCURRENT ILLNESSES)

Parents recorded the presence of local symptoms (swelling or induration of any size) at injection site; systemic symptoms (fever, excessive crying, fretfulness, appetite changes); and major neurological symptoms (hypotonic-hyporesponsive episodes, convulsions) on a specially designed illustrated record sheet (Appendix 1) for a period of 14 days after each vaccination.

Detailed instructions pertaining to interpretation of the record sheet, especially convulsions and hypotonic-hyporesponsive episodes were conveyed in the native language (ZULU) by a registered nurse. Fever was measured by clinical impression of the mother. Before the second and third doses of vaccine, parents were questioned about any untoward effects that had resulted following the previous injection. parents were instructed to inform study personnel immediately of any serious reactions, usually through a registered nurse. Intercurrent illnesses in all infants (between clinic visits) were monitored through home visits by a registered nurse with access to tertiary health care.

11.4.6 SEROLOGIC ASSAYS

Determinations of IgG antibodies to FHA, PT and AGG2,3 were performed by ELISA at the Centre for Applied Microbiology and Research, Public Health Laboratory Service, Porton, UK by the author. The procedure used was essentially as described by Rutter *et al.* (1988) All sera from one individual were tested in the same assay on the same day. Titres were expressed as ELISA U/ml. Results were reported as geometric mean titres (GMTs). Seroconversion was defined as a $\geq$4-fold rise in titre from pre-vaccination titres.

Reference sera: Human IgG Pertussis Serum positive for antibodies to PT, FHA and AGG2,3. Lot 10, supplied by Biken-Kanonji (Osaka, Japan) as a freeze-dried preparation containing 250 ELISA U/ml PT antibody and 400 ELISA U/ml FHA antibody, was used throughout to allow calculation of antibody unitage. This reference sera was assigned a value of 400 ELISA U/ml IgG-anti-AGG2,3. The unitage of the test serum relative to the reference serum was calculated by means of parallel line assays.

Antigens: FHA and PT each containing 200 mg protein nitrogen/ml were supplied by the Biken-Kanonji Institute, Osaka, Japan for use in ELISA. The fimbrial antigen, co-purified AGG2,3 containing 500 μg/ml protein nitrogen was supplied by Dr. A. Robinson, Biologics Division, PHLS, CAMR, Porton, UK as a freeze-dried preparation containing agglutinogens 2 and 3 in a

ratio of 6:4.

11.4.7 TRANSPLACENTAL TRANSFER OF ANTIBODIES

This was estimated for all 3 antibodies (PT, FHA, AGG2,3) from measurements obtained in mother-baby pairs in Groups SN and SP only. The formula used was -

$$T = \frac{Ab_b}{Ab_m}$$

where T = placental transfer
Ab_b = antibody titre in baby
Ab_m = antibody titre in mother

11.4.8 STATISTICAL ANALYSIS

Subjects were classified as SN (SGA infants who were well-nourished subsequently); SP (SGA infants who subsequently developed PEM) or PEM (infants who developed PEM before the age of 9 months) or WN (well-nourished) for the purpose of analysis. The number of subjects in these groups was too small to permit statistical analysis. Calculations on and analysis of antibody titres and ELISA values were performed on logarithmically transformed data. When malnourished infants were grouped together, unpaired t-tests (unequal variances) were used for comparison of geometric mean titres in PEM and well-nourished groups of infants. Fishers' exact test or chi square was used to test differences in proportions of seroconversion, frequency and incidence of post-vaccination events in PEM and well-nourished infants. $P < 0.05$ was considered a statistically significant difference on a 95% confidence level.

11.5 RESULTS

11.5.1 PERTUSSIS INFECTION

Eleven infants with evidence of recent pertussis infection were diagnosed. Ten cases were subclinical and occurred in well-nourished infants; only one of whom had completed the primary vaccination course. These were diagnosed retrospectively on the basis of serologic

evidence and are not included in this part of the study. One male infant (SN) contracted pertussis between 2 and 4 months of age. He presented with cough of > 1 weeks duration and subconjunctival haemorrhage. The subject was underweight at birth and had received the first dose of A-DTP at this time and the second dose at two months of age. The infant had high levels of anti-FHA (290.3 U/ml) and anti-AGG2,3 (130.4 U/ml) but negligible anti-PT (2.0 U/ml) in cord blood. Immediately prior to infection (at two months of age) the anti-PT level was 59.0 U/ml, the anti-FHA 70.2 U.ml and anti-AGG2,3 72.4 U/ml, not significantly different from those of uninfected age-matched cohorts. Incidence of pertussis infection per thousand doses of acellular and whole-cell vaccine is shown in Table 11.2.

11.5.2 INTERCURRENT ILLNESSES

Incidence and nature of intercurrent illnesses from birth to 9 months of age are shown in Table 11.3, 11.4 and 11.5. The frequency of intercurrent illnesses was similar in malnourished infants (SN, SP and NP) and age-matched well-nourished babies (46% vs. 43.2%) ($p > 0.05$). Upper respiratory tract and skin infections were the two most commonly occurring illnesses in both groups (15% vs. 19%; 15% vs. 14%) respectively. In the malnourished group 20/29 (69%) of illnesses occurred in infants ≤6 months of age as compared with 225/248 (49%) in well-nourished infants ($p = 0.05$, chi square). SN infants did not experience a significantly different number of illnesses compared with SP or NP infants ($p > 0.05$).

11.5.3 POST-VACCINATION EVENTS

Frequency of post-vaccination events following administration of acellular and whole-cell vaccines in malnourished infants are shown in Tables 11.6, 11.7 and 11.8.

Major events: No major neurologic event occurred amongst SN, SP or NP infants. In well-nourished cohorts the rate of convulsions was 1.69/1000 doses in recipients of A-DTP and 4.02/1000 doses in recipients of W-DTP. Hypotonic-hyporesponsive episodes occurred at a rate of 8.03/1000 in well-nourished recipients of W-DTP.

Minor events: In Group I, one SP infant experienced fever and crying at 2 months.

In Group II, 2 NP infants experienced a total of 4 minor symptoms at 4 months of age after two doses of A-DTP. The first infant was malnourished at two and at four months of age and experienced induration at injection site and excessive crying; the second infant was malnourished at 4 months of age and experienced fever and irritability. A third infant (SP) who developed PEM at 2 and 9 months experienced loss of appetite at 2 months.

One 4 month old infant from Group III, who had been malnourished at 2 months of age (NP), experienced fever after the second dose of A-DTP. No significant differences were noted in the incidence of any minor post-vaccination symptom in PEM (SN, + SP + NP) as compared with well nourished infants (Fishers' Exact Test).

11.5.4 TRANSPLACENTAL TRANSFER OF ANTIBODY

Maternal and cord pertussis IgG antibody levels in SGA and appropriate-for-gestational-age (AGA) infants are shown in Table 11.9.

PT and AGG2,3: SGA infants and their mothers had significantly lower levels of IgG-PT ($t=2.5661$, $p=0.0120$; $t=3.0865$, $p=0.0029$) respectively and IgG-AGG2,3 ($t=2.2235$, $p=0.0276$; $t=2.3067$, $p=0.0219$) respectively compared with well-nourished cohorts.

FHA: IgG-FHA was marginally lower ($p=0.05$) in mothers of SGA infants, however no significant difference in cord blood IgG-FHA was noted in comparison with appropriate controls.

The cord/maternal ratio of IgG-FHA, PT and AGG2,3 was similar in SGA and AGA groups of infants ($p > 0.05$); indicating that the mechanism and rate of transfer of pertussis antibodies was not altered in SGA infants.

11.5.5 SEROLOGICAL RESPONSES TO VACCINATION

SN INFANTS:

Peak and final IgG-PT, FHA and AGG2,3 antibody levels in SN (SGA infants who were subsequently well-nourished) following acellular and whole-cell vaccines are shown in Tables 11.10, 11.11 and 11.12.

Group I (N=1)

PT & FHA: Peak IgG-PT and IgG-FHA titres in 1 SN infant who received a neonatal dose of A-DTP was lower than that in well-nourished (WN) infants. The age at which peak anti-PT and anti-FHA titres occurred (6 months) was delayed until 9 months in this infant.

AGG2,3: Peak IgG-AGG2,3 titres were however considerably higher than in the WN group. The age at which peak anti-AGG2,3 titres occurred (2 months) was similar in both WN and SN infants.

Group II (N=4)

PT: Peak anti-PT titres were lower in 3 SN infants and higher in 1 infant than those attained in the WN group. Final anti-PT titres in 3 infants for whom data was available was considerably lower than in the WN group. The age at of peak anti-PT titres occurred in the WN group (6 months) was delayed until 9 months in 1 of the 4 SN infants.

FHA: Peak anti-FHA titres were considerably lower in all 4 SN infants. The age of peak anti-FHA (9 months) occurred earlier in 1 of the 4 SN infants (6 months).

AGG2,3: Peak anti-AGG2,3 titres were higher in 2 SN infants and lower in 2 SN infants compared with WN infants. Peak anti-AGG2,3 titres occurred earlier in 2 of the 4 SN infants (at 4 and 6 months) than in WN infants (9 months).

Group III (N=3)

PT & FHA: Peak anti-PT titres were higher than that of WN infants in all 3 SN infants. Final titres however were lower in 2 SN infants and higher in 1 SN infant. Peak anti-FHA titres were lower than that of WN infants in all 3 SN infants. Final titres were considerably lower in 2 SN infants and slightly higher in the third. The age at which peak anti-PT and anti-FHA titres occurred (2 months) was similar in WN and SN infants.

AGG2,3: Peak anti-AGG2,3 titres were considerably higher in 2 SN infants and lower in 1 SN infant. The age at which peak anti-AGG2,3 titres were attained (9 months) occurred earlier (at 6 months) in 2 of the 3 SN infants.

SP INFANTS:

IgG-PT, FHA and AGG2,3 titres in SP infants (who developed PEM before completion of the primary vaccination course) are shown in Tables 11.13, 11.14 and 11.15.

Group I (N=2)

PT: The peak anti-PT titre was higher in 1 SP infant and lower in the second infant compared with well-nourished cohorts. For the 1 SP infant for whom data was available the final anti-PT titre at 9 months was considerably lower than in the control group. The age at which peak anti-PT levels were attained in well-nourished infants, ie. 6 months, was the same in 1 SP infant and occurred earlier (at 2 months) in the second, ie. the infant did not respond to PT.

FHA: Peak anti-FHA titres were lower than the GMT of well-nourished infants in both SP infants. Final anti-FHA titres were higher than the GMT of well-nourished infants in 1 case and lower in the second case. The age at which peak anti-FHA levels were attained, ie. 6 months, was similar in well and malnourished (SN) infants.

AGG2,3: Peak anti-AGG2,3 levels were higher than the GMT of well-nourished cohorts in 1

infant and lower in the second infant. The age at which peak AGG2,3 antibody titres were attained, ie. 9 months, was similar in well and malnourished (SP) infants.

Group II (N = 4)

PT: Peak anti-PT titres were lower in all 4 SP infants compared with GMTs of well-nourished infants. In addition, 2 of these infants had final anti-PT titres which were considerably lower than that of well-nourished infants. The age at which peak anti-PT were attained (6 months) was delayed until 9 months in 2 of the 4 SP infants.

FHA: Peak anti-FHA titres were considerably lower in all 4 SP infants. In 1 SP infant for whom data was available, final titres were considerably lower than in the well-nourished group. The age of peak anti-FHA attainment in well-nourished infants (9 months) occurred earlier in 1 SP infant (at 6 months) and at 9 months in the other 3.

AGG2,3: Peak anti-AGG2,3 titres were lower in all 4 SP infants than in well-nourished cohorts. The age at which peak titres were attained was not affected by nutritional status.

Group III (N = 3)

PT: Peak and final anti-PT titres were considerably lower in 2 SP infants; and peak titres higher in the third infant, compared with well-nourished infants. The age at which peak anti-PT titres were attained in WN infants (2 months) was delayed in all 3 SP infants; until 6 months in 2 infants and until 9 months in the third.

FHA: Peak anti-FHA titres were lower in all 3 SP infants compared with WN infants. In addition, final titres were considerably lower in 1 of these infants. The age at which peak titres were attained in the WN group (2 months) was delayed until 9 months in 2 SP infants, but was not altered in the third.

AGG2,3: Peak anti-AGG2,3 titres were lower in all 3 SP infants compared with WN infants. In

addition, final titres were considerably lower in 1 of these (SP) infants. Peak anti-AGG2,3 titres were attained at 9 months in well-nourished infants and in 2 SP infants, and earlier at 6 months in the third SP infant.

NP:

Peak and final IgG-PT, FHA and AGG2,3 antibody levels in NP (well-nourished recipients of acellular and whole-cell pertussis vaccine who developed PEM before the age of 9 months) are shown in Tables 11.16, 11.17 and 11.18.

Group I (N=2)

PT: Peak and final anti-PT titres were considerably lower in NP compared with WN infants. In 1 of the 2 NP infants, the age at which peak titres were attained in controls (6 months) was delayed until 9 months.

FHA: Peak and final anti-FHA titres were considerably lower in NP compared with WN infants. The age at which peak titres were attained was similar in both groups.

AGG2,3: Peak and final anti-AGG2,3 titres were higher in 1 of the 2 NP infants compared with controls. Peak titres were attained 3 months earlier (at 6 months) in this infant.

Group II (N=4)

PT: Both peak and final anti-PT titres were considerably higher in NP compared with well-nourished infants. The age at which peak titres were attained was however delayed from 6 until 9 months in 2 of the 4 NP infants.

FHA: Peak anti-FHA titres were considerably lower in 3 of the 4 NP infants compared with controls. The age at which peak titres were attained in WN infants (9 months) was the same as in 3 of the 4 NP infants.

AGG2,3: Peak anti-AGG2,3 titres were higher and attained at an earlier age in 3 of the 4 NP infants, compared with controls.

Group III (N=2)

PT and FHA: Peak and final anti-PT and anti-FHA titres were lower in NP compared with controls. Infants with NP attained peak anti-PT and anti-FHA titres later (at 4 and 9 months) than well-nourished infants (at 2 months).

AGG2,3: Peak AGG2,3 titres were higher in 1 of the 2 NP infants compared with controls. The age at which peak titres were attained were similar in both groups.

When SN, SP and NP infants were analysed as a single group (N=25) peak and final titres of anti-PT and peak anti-FHA titres were found to be similar overall in mal- and well-nourished recipients of A-DTP (although often lower in the former); final anti-FHA values were significantly lower ($p=0.035$) in NP. In recipients of W-DTP, no significant differences were noted between peak and final titres of all three antibodies in malnourished and well-nourished infants (Tables 11.19, 11.20 and 11.21).

Frequency and percentage seroconversion to all 3 antibodies measured was not significantly different in malnourished and well-nourished infants in any vaccine group ($p > 0.05$) (Table 11.22). The nature of the antibody response depended on the type of vaccine administered. Recipients of A-DTP (Groups I and II) produced a substantial response to PT and FHA, but not to AGG2,3, irrespective of nutritional status. Both malnourished and well-nourished recipients of W-DTP (Group III) did not respond to PT and FHA, although the rate of AGG2,3 seroconversion was 62.5%.

11.6 DISCUSSION

The presence of infection and the age of onset of nutritional deprivation, amongst other factors, have been shown to compromise the immune response to vaccination (Chandra, 1972, 1979).

PEM and LBW are common in developing countries and may in some way affect the response to vaccination. Although the currently used whole-cell DTP vaccine appears to be effective in the Third World, the pertussis component of this vaccine has not been separately evaluated. There is little data on the immune response to whole-cell pertussis vaccination in post- and peri-natal malnutrition; nor has the safety and immunogenicity of acellular pertussis vaccines in these infants been established.

The only other investigation of acellular pertussis vaccine in a developing country (Biritwum *et al.*, 1985) found no relationship between vaccine-induced antibody levels and nutritional status in 110 children aged three months to three years, 25% of whom had body weights of <80% of the WHO standard for their age, and 3% of whom were severely wasted.

Intra-uterine growth retardation or post-natal nutritional deprivation did not appear to affect the response to acellular or whole-cell pertussis vaccination. Nevertheless the small number of malnourished infants calls for caution in making a definite statement.

The majority of recipients of A-DTP in the present study had a definite immune response to PT and FHA by nine months of age, irrespective of levels of maternally acquired pertussis antibodies or nutritional status. Malnourished infants responded no less well to acellular pertussis vaccination than did well-nourished age and vaccine matched cohorts. In both malnourished and well-nourished infants, acellular DTP elicited a better serologic response to FHA and PT when administered according to routine vaccination schedules commenced at 2 months than when vaccination commenced at birth. This muted response may be attributed to the well-known inhibitory effect of maternally-derived antibodies. The response to AGG2,3 in

recipients of acellular DTP was, not surprisingly, significantly lower than in whole-cell vaccine recipients, since the former preparation contained only a negligible amount of agglutinogens.

In contrast with the serum antibody responses to PT and FHA reported in other series of whole-cell vaccine (Edwards *et al.*, 1986; Baraff *et al.*, 1984; Ashworth *et al.*, 1983) recipients of this vaccine in the present study did not respond to these antigens irrespective of nutritional status, which questions the immunogenicity of the South African product.

The incidence of adverse effects to vaccination in general have not been reported to be increased in mild to moderate PEM. The WHO does not consider malnutrition to be a contraindication for the administration of measles, BCG or oral polio vaccines to children (Galazka *et al.*, 1984). No statistical differences in the rate of minor post-vaccination symptoms in malnourished and well-nourished infants was noted in the present study.

It was widely recognised that PEM in general leads to increased frequency and severity of illnesses. The incidence of illness in infants aged six months or younger in the present study was significantly higher in PEM.

Of the eleven cases of pertussis infection that occurred during the study period, ten were asymptomatic and occurred in well-nourished infants. Clinical signs of pertussis were observed in one four month old infant after two doses of vaccine who had been SGA at the time of administration of the first dose of A-DTP. Intra-uterine nutritional deprivation did not appear to inhibit response to vaccination in this infant. Antibody levels of IgG-PT, IgG-FHA and IgG-AGG2,3 after the first dose of acellular DTP were comparable with those attained in well-nourished cohorts who remained uninfected. The acquisition of IgG antibodies to PT and FHA has not however been shown to correlate with clinical immunity in several studies (Long *et al.*, 1990a,b). The susceptibility of this SGA infant to pertussis may be connected with a lack of pertussis IgA antibody, which is thought to be of importance in protective immunity (Pittman,

1976). No maternal or secretory IgA is present in the newborn and although serum IgG and IgM levels in PEM are normal, secretory IgA levels are known to be low and may therefore predispose SGA infants to infection.

It is interesting to note the significant pertussis antibody levels in maternal and cord sera. Most First World studies have shown a lack of demonstrable antibodies in women of child-bearing age (Brown 1960; Burstyn *et al.*, 1983) Since these antibodies are unlikely to be due to the South African whole-cell vaccine, given the poor antibody response to PT and FHA in the present study, it is presumed that these antibodies are the end result of natural infection and that pertussis is widespread in the African Community.

Black (1988) found maternally acquired measles antibody levels to be higher in developing countries than in the West and that malnutrition had no major effect on the transplacental transfer of measles antibody in developing countries. Findings in this study confirm the above with respect to pertussis antibodies. Significantly lower levels of PT and FHA antibodies were detected in mothers of SGA infants in comparison with mothers of well-nourished infants; circulating maternal antibody was hence correspondingly lower in these infants. The mechanism and rate of transplacental transfer was not found to be compromised in SGA infants.

TABLE 11.1 Nutritional indices at different ages during the study in 25 African infants with protein energy malnutrition vaccinated with acellular or whole-cell pertussis vaccine.

	AGE IN MONTHS														
	Weight/Age < 3rd Centile					Length/Age < 10th Centile					Weight/Age & Length/Age < 3rd Centile				
	0	2	4	6	9	0	2	4	6	9	0	2	4	6	9
Group I															
Case 1	+														
Case 2						+									+
Case 3						+									+
Case 4	+														
Case 5	+														
Group Ii															
Case 1	+														
Case 2	+														
Case 3						+									
Case 4						+									
Case 5											+	+	+		
Case 6						+									+
Case 7						+									+
Case 8						+									+
Case 9		+													
Case 10					+										
Case 11												+	+		
Case 12															+
Group III															
Case 1						+									
Case 2											+				
Case 3											+				
Case 4	+													+	
Case 5	+			+											
Case 6														+	
Case 7				+											
case 8					+										

Group I Acellular-DTP from birth.
Group II Acellular-DTP from 2 months.
Group III Whole-cell DTP from 2 months.

TABLE 11.2 Incidence of pertussis infection in malnourished and well-nourished recipients of acellular and whole-cell pertussis vaccine.

Vaccine	INCIDENCE/1000 DOSES (Number of cases) Well-nourished	Malnourished
Acellular-DTP	11.84 (7)	1.69 (1)
Whole-cell DTP	12.05 (2)	0

TABLE 11.3 Intercurrent illnesses from 0 to 9 months of age in African infants with PEM* and in age-matched infants of normal nutritional status given either acellular or whole-cell pertussis vaccines.

TYPE OF INFECTION	% OF CHILDREN WITH INFECTION	
	Malnourished infants (N=25)	Well-nourished infants (N=317)
Upper respiratory tract	15	19
Skin	15	14
Diarrhoea	7	5
Lower respiratory tract	3	4
Eye	3	1
Chicken pox	3**	0.2
TOTAL	46	43.2

* Including SN, SP and NP infants
** $p < 0.05$

TABLE 11.4 intercurrent illness in malnourished black infants from birth to 9 months of age.

	NUMBER OF ILLNESSES						
	URTI*	Skin	Diarrhoea	LRTI*	Eye	Chicken pox	Total
AGE GROUP (months)							
0≤2							
SN	1						1
SP	1	1					2
NP					1		1
>2≤4							
SN				1			1
SP	1	2					3
NP		1	1				2
>4≤6							
SN	2	1					3
SP	2		1			1	4
NP	1	1		1			3
>6≤9							
SNA	1	2			1		4
SP	1	1	1				3
NP		1				1	2

* URTI, LRTI: upper or lower respiratory tract infection.

SN Small-for-gestation-age (N=8) who were subsequently well-nourished.
SP SGA infants who subsequently developed PEM (N=8).
NP Infants who developed PEM by 9 months (N=9).

TABLE 11.5 Incidence of intercurrent illnesses in protein-energy malnutrition (PEM) and in well-nourished infants according to age.

AGE RANGE (months)	NUMBER OF ILLNESSES SN	SP	NP	All Malnourished infants*	Normal Nutritional Status
0≤2	1	2	1	4	23
>2≤4	1	3	2	6	98
>4≤6	3	4	3	10	109
≤6	5	9	6	20	225
>6≤9	4	3	2	9	223
TOTAL	9(31%)	12(41%)	8(28%)	29	448

SN Small-for-gestational age (SGA) infants who became well-nourished by 2 months of age.
SP SGA infants who subsequently developed PEM.
NP Well-nourished infants who developed PEM by 9 months of age.

* SN + SP + NP.

TABLE 11.6 Frequency of post-vaccination events (PVE) following administration of acellular pertussis vaccine commenced at birth in malnourished and well-nourished infants (Group I).

	NUMBER OF PVE				
	SN	SP	NP	All malnourished infants (20 doses)	Well-nourished* infants (349 doses)
Major					
Convulsions	0	0	0	0	1
Hypotonic-hyporesponsive episodes	0	0	0	0	0
Systemic					
Excessive crying	0	1	0	1	10
Fever	0	1	0	1	8
Irritability	0	0	0	0	7
Loss of appetite	0	0	0	0	2
Local (at injection site)					
Induration	0	0	0	0	1
Swelling	0	0	0	0	1

* Age and vaccine matched infants

SN Small-for-gestational age (SGA) infants who were well-nourished by 2 months of age.
SP SGA infants who developed PEM before completion of the primary vaccination course, ie. by 9 months of age.
NP Well-nourished infants who developed PEM by 9 motnhs of age.

No statistically significant difference occurred in the number of post-vaccination events recorded in malnourished infants compared with well-nourished cohorts (Fishers' Exact Test).

TABLE 11.7 Frequency of post-vaccination events (PVE) following administration of acellular pertussis vaccine in malnourished and well-nourished infants (Group II).

	NUMBER OF PVE				
	SN	SP	NP	All malnourished infants (36 doses)	Well-nourished* infants (242 doses)
Major					
Convulsions	0	0	0	0	0
Hypotonic-hyporesponsive episodes	0	0	0	0	0
Systemic					
Excessive crying	0	1	1	2	15
Fever	0	0	1	1	10
Irritability	0	1	1	2	2
Loss of appetite	0	1	0	1	3
Local (at injection site)					
Induration	0	0	0	0	2
Swelling	0	0	1	1	1

* Age and vaccine matched infants

SN Small-for-gestational age (SGA) infants who were well-nourished by 2 months of age.
SP SGA infants who developed PEM before completion of the primary vaccination course, ie. by 9 months of age.
NP Well-nourished infants who developed PEM by 9 motnhs of age.

No statistically significant difference in the rates of occurrence of PVE in malnourished and well-nourished infants was noted ($p > 0.05$).

TABLE 11.8 Frequency of post-vaccination events (PVE) following administration of whole-cell pertussis vaccine in malnourished and well-nourished infants (Group III).

	NUMBER OF PVE				
	SN	SP	NP	All malnourished infants (24 doses)	Well-nourished* infants (249 doses)
Major					
Convulsions	0	0	0	0	1
Hypotonic-hyporesponsive episodes	0	0	0	0	2
Systemic					
Excessive crying	0	0	0	0	6
Fever	0	0	1	1	8
Irritability	0	0	0	0	1
Loss of appetite	0	0	0	0	1
Local (at injection site)					
Induration	0	0	0	0	0
Swelling	0	0	0	0	0

* Age and vaccine matched infants

SN Small-for-gestational age (SGA) infants who were well-nourished by 2 months of age.
SP SGA infants who developed PEM before completion of the primary vaccination course, ie. by 9 months of age.
NP Well-nourished infants who developed PEM by 9 motnhs of age.

No statistically significant difference in the rates of occurrence of PVE in malnourished and well-nourished infants was noted ($p > 0.05$).

TABLE 11.9 Transplacental transfer of pertussis IgG antibodies in small-for-gestational-age (SGA) and appropriate-for-gestational-age (AGA) infants.

ANTIBODY	ELISA U/ml SGA	AGA	P-VALUE
IgG-PT			
Maternal	26.0 ± 4.9	45.7 ± 5.9	0.01
Cord	24.0 ± 5.0	46.8 ± 5.5	0.003
Cord/maternal	1.0 ± 0.1	2.0 ± 0.6	0.14
IgG-FHA			
Maternal	55.7 ± 10.1	79.4 ± 6.3	0.05
Cord	77.1 ± 22.0	87.1 ± 8.9	0.67
Cord/maternal	1.3 ± 0.2	1.3 ± 0.1	0.87
IgG-AGG2,3			
Maternal	156.2 ± 46.3	367.3 ± 82.9	0.03
Cord	97.9 ± 27.6	366.9 ± 113.3	0.02
Cord/maternal	0.88 ± 0.14	1.09 ± 0.10	0.22

TABLE 11.10 IgG-PT antibody levels in small-for-gestational age (SN) infants following acellular and whole-cell pertussis vaccines (ELISA U/ml).

VACCINE GROUP	PEAK TITRE	AGE AT PEAK (MONTHS)	FINAL TITRE*
Group I			
Case 1	87.7	9	-
Well-nourished cohorts	138.0 ± 23.2** (N=60)	6	117.0 ± 19.4 (N=52)
Group II			
Case 1	132.1	6	73.9
Case 2	123.6	6	95.2
Case 3	250.7	9	-
Case 4	23.5	6	18.6
Well-nourished cohorts	183.4 ± 23.0 (N=68)	6	136.4 ± 15.5 (N=67)
Group III			
Case 1	76.6	2	1.2
Case 2	49.7	2	38.5
Case 3	39.9	2	1.0
Well-nourished cohorts	31.7 ± 10.3 (N=49)	2	18.4 ± 4.2 (N=45)

* At 9 months

** Geometric mean ± standard error

Group I Acellular DTP from birth

Group II Acellular DTP from 2 months

Group III Whole-cell DTP from 2 months

TABLE 11.11 IgG-FHA antibody levels in small-for-gestational age (SN) infants following acellular and whole-cell pertussis vaccines (ELISA U/ml).

VACCINE GROUP	PEAK TITRE	AGE AT PEAK (MONTHS)	FINAL TITRE*
Group I			
Case 1	117.8	9	-
Well-nourished cohorts	145.5 ± 52.8** (N=59)	6	84.5 ± 11.2 (N=50)
Group II			
Case 1	138.8	9	-
Case 2	300.3	9	-
Case 3	238.8	6	172.4
Case 4	17.2	9	-
Well-nourished cohorts	486.0 ± 14.3 (N=49)	9	-
Group III			
Case 1	40.6	2	7.4
Case 2	78.3	2	65.4
Case 3	53.9	2	30.5
Well-nourished cohorts	82.8 ± 46.0 (N=50)	2	60.8 ± 12.5 (N=50)

* At 9 months

** Geometric mean ± standard error

Group I Acellular DTP from birth

Group II Acellular DTP from 2 months

Group III Whole-cell DTP from 2 months

TABLE 11.12 IgG-AGG2,3 antibody levels in small-for-gestational age (SN) infants following acellular and whole-cell pertussis vaccines (ELISA U/ml).

VACCINE GROUP	PEAK TITRE	AGE AT PEAK (MONTHS)	FINAL TITRE*
Group I			
Case 1	295.3	9	-
Well-nourished cohorts	138.3 ± 29.6** (N=49)	9	-
Group II			
Case 1	41.9	4	16.8
Case 2	398.3	9	-
Case 3	239.4	6	51.6
Case 4	29.5	9	-
Well-nourished cohorts	94.0 ± 22.3 (N=50)	9	-
Group III			
Case 1	5149.2	9	-
Case 2	4362.1	6	3116.4
Case 3	142.8	6	142.8
Well-nourished cohorts	714.3 ± 160.2 (N=44)	9	-

* At 9 months
** Geometric mean ± standard error

Group I Acellular DTP from birth
Group II Acellular DTP from 2 months
Group III Whole-cell DTP from 2 months

TABLE 11.13 IgG-PT antibody levels in small-for-gestational age infants who developed protein-energy-malnutrition during primary vaccination (SP) with acellular or whole-cell pertussis vaccines (ELISA U/ml).

VACCINE GROUP	PEAK TITRE	AGE AT PEAK (MONTHS)	FINAL TITRE*
Group I			
Case 1	321.6	6	-
Case 2	53.6	2	27.2
Well-nourished cohorts	138.0±23.2** (N=60)	6	117.0±19.4 (N=52)
Group II			
Case 1	55.5	6	41.7
Case 2	149.3	9	-
Case 3	39.0	9	-
Case 4	85.2	6	69.5
Well-nourished cohorts	183.0±23.0 (N=68)	6	136.4±15.5 (N=67)
Group III			
Case 1	2.3	6	0.9
Case 2	36.2	9	-
Case 3	4.6	6	4.4
Well-nourished cohorts	31.7±10.3 (N=49)	2	18.4±4.2 (N=45)

* At 9 months

** Geometric mean ± standard error

Group I Acellular DTP from birth
Group II Acellular DTP from 2 months
Group III Whole-cell DTP from 2 months

TABLE 11.14 IgG-FHA antibody levels in small-for-gestational age infants who developed protein-energy-malnutrition during primary vaccination (SP) with acellular or whole-cell pertussis vaccines (ELISA U/ml).

VACCINE GROUP	PEAK TITRE	AGE AT PEAK (MONTHS)	FINAL TITRE*
Group I			
Case 1	136.7	6	97.9
Case 2	59.9	6	19.9
Well-nourished cohorts	145.5 ± 52.8** (N=59)	6	84.5 ± 11.2 (N=50)
Group II			
Case 1	47.3	6	46.8
Case 2	65.8	9	-
Case 3	31.2	9	-
Case 4	48.5	9	-
Well-nourished cohorts	486.0 ± 14.3 (N=49)	9	486.0 ± 14.3 (N=49)
Group III			
Case 1	7.0	2	3.7
Case 2	61.1	9	-
Case 3	14.7	9	-
Well-nourished cohorts	82.8 ± 46.0 (N=50)	2	60.8 ± 12.5 (N=50)

* At 9 months

** Geometric mean ± standard error

Group I Acellular DTP from birth
Group II Acellular DTP from 2 months
Group III Whole-cell DTP from 2 months

TABLE 11.15 IgG-AGG2,3 antibody levels in small-for-gestational age infants who developed protein-energy-malnutrition during primary vaccination (SP) with acellular or whole-cell pertussis vaccines (ELISA U/ml).

VACCINE GROUP	PEAK TITRE	AGE AT PEAK (MONTHS)	FINAL TITRE*
Group I			
Case 1	784.6	9	-
Case 2	56.3	9	-
Well-nourished cohorts	138.0 ± 29.6** (N = 49)	9	-
Group II			
Case 1	84.5	9	-
Case 2	69.2	9	-
Case 3	58.5	9	-
Case 4	90.0	6	-
Well-nourished cohorts	94.0 ± 22.3 (N = 50)	9	-
Group III			
Case 1	193.2	6	93.5
Case 2	503.7	9	-
Case 3	56.5	9	-
Well-nourished cohorts	714.3 ± 160.2 (N = 44)	9	714.3 ± 160.2 (N = 44)

* At 9 months
** Geometric mean ± standard error

Group I Acellular DTP from birth
Group II Acellular DTP from 2 months
Group III Whole-cell DTP from 2 months

TABLE 11.16 IgG-PT antibody levels in african infants who developed protein-energy-malnutrition (NP) during primary vaccination with acellular or whole-cell pertussis vaccine (ELISA U/ml).

VACCINE GROUP	PEAK TITRE	AGE AT PEAK (MONTHS)	FINAL TITRE*
Group I			
Case 1	62.3	6	37.1
Case 2	43.9	9	-
Well-nourished cohorts	138.0 ± 23.2** (N=60)	6	117.0 ± 19.4 (N=52)
Group II			
Case 1	295.1	9	-
Case 2	280.1	9	-
Case 3	970.9	6	307.8
Case 4	317.6	6	140.5
Well-nourished cohorts	183.4 ± 23.0 (N=68)	6	136.4 ± 15.5 (N=67)
Group III			
Case 1	8.9	4	4.2
Case 2	9.6	9	-
Well-nourished cohorts	31.7 ± 10.3 (N=49)	2	18.4 ± 4.2 (N=45)

* At 9 months

** Geometric mean ± standard error

Group I Acellular DTP from birth
Group II Acellular DTP from 2 months
Group III Whole-cell DTP from 2 months

TABLE 11.17 IgA-FHA antibody levels in infants who developed protein-energy malnutrition (NP) during primary vaccination with acellular or whole-cell pertussis vaccine (ELISA U/ml).

VACCINE GROUP	PEAK TITRE	AGE AT PEAK (MONTHS)	FINAL TITRE*
Group I			
Case 1	74.4	6	47.9
Case 2	39.6	6	37.4
Well-nourished cohorts	145.5 ± 52.8** (N=59)	6	84.5 ± 11.2 (N=50)
Group II			
Case 1	140.3	9	-
Case 2	42.1	9	-
Case 3	607.9	6	189.1
Case 4	130.1	9	-
Well-nourished cohorts	486.0 ± 14.3 (N=49)	9	486.0 ± 14.3 (N=49)
Group III			
Case 1	16.0	9	-
Case 2	38.1	4	26.4
Well-nourished cohorts	82.8 ± 46.0 (N=50)	2	60.8 ± 12.5 (N=50)

* At 9 months
** Geometric mean ± standard error

Group I Acellular DTP from birth
Group II Acellular DTP from 2 months
Group III Whole-cell DTP from 2 months

TABLE 11.18 IgG-AGG2,3 antibody levels in infants who developed protein-energy malnutrition (NP) during primary vaccination with acellular or whole-cell pertussis vaccine (ELISA U/ml).

VACCINE GROUP	PEAK TITRE	AGE AT PEAK (MONTHS)	FINAL TITRE*
Group I			
Case 1	163.4	6	159.8
Case 2	92.3	9	-
Well-nourished cohorts	138.3 ± 29.6** (N = 49)	9	138.3 ± 29.6 (N = 49)
Group II			
Case 1	107.2	6	28.5
Case 2	40.9	9	-
Case 3	192.8	6	129.7
Case 4	481.5	6	327.2
Well-nourished cohorts	94.0 ± 22.3 (N = 50)	9	-
Group III			
Case 1	35.9	9	-
Case 2	1079.5	9	-
Well-nourished cohorts	714.3 ± 160.2 (N = 44)	9	-

* At 9 months
** Geometric mean ± standard error

Group I Acellular DTP from birth
Group II Acellular DTP from 2 months
Group III Whole-cell DTP from 2 months

TABLE 11.19 IgG-anti-PT levels following acellular and whole-cell pertussis vaccination in protein-energy malnutrition (SM. SP and NP) and well-nourished (WN) infants.

GROUP I		N	GMT ± SE*	SD**	RANGE	UNPAIRED T-TEST***
Group I						
Peak level:	PEM	5	113.8 ± 52.5	117.3	43.9-321.6	t = -0.4209;
	WN	60	138.0 ± 23.2	179.8	2.0-903.0	p = 0.6893
Final level:	PEM	5	103.5 ± 55.5	124.1	27.2-321.6	6 = -0.2303;
	WN	52	117.0 ± 19.5	140.3	1.5-879.0	p = 0.8270
Group II						
Peak level:	PEM	12	226.9 ± 73.9	256.1	23.5-970.9	t = 0.5609;
	WN	68	183.4 ± 23.0	189.9	12.0-970.9	p = 0.5842
Final level:	PEM	12	226.9 ± 31.4	256.1	18.6-307.8	t = 0.2962;
	WM	57	183.4 ± 15.5	189.9	10.4-136.4	p = 0.7707
Group III						
Peak level:	PEM	8	28.5 ± 9.4	26.6	2.3-76.6	t = 0.4360;
	WM	57	23.4 ± 6.9	52.4	1.0-29.0	p = 0.6686
Final level:	PEM	8	7.5 ± 4.6	12.9	0.9-38.5	t = -1.7612;
	WM	45	18.4 ± 4.2	28.4	1.2-186.2	p = 0.0923

* Geometric mean titre ± standard error
** Standard deviation
*** Unequal variances

Group I Acellular vaccine from birth
Group II Acellular vaccine from 2 months of age
Group III Whole-cell vaccine from 2 months of age

TABLE 11.20 IgG-anti-FHA levels following acellular and whole-cell pertussis vaccination in PEM$^+$ and well-nourished (WN) infants.

GROUP I		N	GMT ± SE*	SD**	RANGE	UNPAIRED T-TEST***
Group I						
Peak level:	PEM	5	85.7 ± 18.1	40.5	39.6-136.7	t = -1.0716;
	WN	59	145.5 ± 52.8	405.7	5.7-3120.0	p = 0.2882
Final level:	PEM	5	64.2 ± 18.6	41.7	19.9-117.8	t = -0.9331;
	WN	50	84.5 ± 11.2	79.2	11.0-485.9	p = 0.3806
Group II						
Peak level:	PEM	12	150.7 ± 48.6	168.4	17.2-607.9	t = 0.8640;
	WN	59	107.2 ± 12.9	99.5	14.2-607.9	p = 0.8138
Final level:	PEM	12	110.2 ± 24.3	99.5	14.2-607.9	t = -0.2387;
	WM	49	116.9 ± 14.3	99.8	9.7-486.0	p = 0.8138
Group III						
Peak level:	PEM	8	38.7 ± 8.9	25.1	7.0-78.3	t = -1.3667;
	WM	60	62.5 ± 15.0	116.1	0.9-664.1	p = 0.1775
Final level:	PEM	8	28.2 ± 8.3	23.4	3.7-65.4	t = -2.1764
	WM	50	60.8 ± 12.5	88.6	0.9-46.0	p = 0.0350#

* Geometric mean titre ± standard error
** Standard deviation
*** Unequal variances
\# $p < 0.05$ denotes a statistically significant difference
\+ PEM: SN + SP + NP

Group I Acellular vaccine from birth
Group II Acellular vaccine from 2 months of age
Group III Whole-cell vaccine from 2 months of age

TABLE 11.21 IgG-anti-AGG2,3 levels following acellular and whole-cell pertussis vaccination in PEM^+ and well-nourished (WN) infants (ELISA U/ml).

GROUP I		N	GMT ± SE*	SD**	RANGE	UNPAIRED T-TEST
Group I						
Peak level:	PEM	5	278.4 ± 133.0	297.4	56.3-784.6	t = 0.5598;
	WN	58	200.3 ± 42.1	320.9	0.9-1650.1	p = 0.6006
Final level:	PEM	5	277.7 ± 133.1	297.7	56.3-784.6	t = 0.4489;
	WN	49	214.4 ± 46.0	321.7	0.9-1426.0	p = 0.6723
Group II						
Peak level:	PEM	12	152.8 ± 43.0	148.9	29.5-481.5	t = 1.0917;
	WN	62	103.0 ± 15.3	120.8	0.9-605.3	p = 0.2935
Final level:	PEM	12	110.4 ± 35.5	122.9	16.8-398.3	t = -0.7118;
	WN	50	145.7 ± 34.6	245.0	0.9-1597.1	p = 0.4813
Group III						
Peak level:	PEM	8	1440.4 ± 737.1	2084.9	35.9-5149.2	t = 0.9059;
	WN	54	754.7 ± 171.7	1261.7	0.9-6681.0	p = 0.3923
Final level:	PEM	8	1272.2 ± 664.6	1879.6	35.9-5149.2	t = 0.2326;
	WM	44	1107.2 ± 248.0	1645.1	0.9-8968.0	p = 0.8212

* Geometric mean titre ± standard error
** Standard deviation
\# $p < 0.05$ denotes a statistically significant difference
$^+$ PEM: SN + SP + NP

Group I Acellular vaccine from birth
Group II Acellular vaccine from 2 months of age
Group III Whole-cell vaccine from 2 months of age

TABLE 11.22 Frequency and percentage seroconversion* in malnourished infants vaccinated with acellular or whole-cell pertussis vaccines.

VACCINE GROUP	SEROCONVERSION (%) IgG-PT	IgG-FHA	IgG-AGG2,3
Group I			
SN (N=1)	0(0)	0(0)	0(0)
SP (N=2)	2(100)	1(50)	0(0)
NP (N=2)	2(100)	1(50)	0(0)
All malnourished infants#(N=5)	4(80)	2(40)	0(0)
Well-nourished cohorts(N=85)	46(54.1)	20(23.3)	19(22.6)
Group II			
SN (N=4)	3(75)	2(50)	0(0)
SP (N=4)	2(50)	2(50)	0(0)
NP (N=4)	4(100)	3(75)	0(0)
All malnourished infants#(N=12)	9(75)	7(58.3)	0(0)
Well-nourished cohorts(N=86)	52(75.4)	45(69.2)	15(22.1)
Group III			
SN (N=3)	0(0)	0(0)	3(100)
SP (N=3)	0(0)	0(0)	1(33.3)
NP (N=2)	0(0)	0(0)	1(50)
All malnourished infants#(N=8)	0(0)	0(0)	5(62.5)
Well-nourished cohorts(N=84)	11(19.6)	14(24.1)	37(67.3)

* ≥ 4-fold rise in titre from pre-vaccination titres

\# No statistically significant difference was noted between seroconversion in malnourished and well-nourished infants (Fishers' Exact Test)

SP SGA infants who developed PEM before completion of the primary vaccine course

SN SGA infants who were subsequently well-nourished

NP Well-nourished infants who subsequently developed PEM

Group I Acellular DTP from birth

Group II Acellular DTP from 2 months

Group III Whole-cell DTP from 2 months

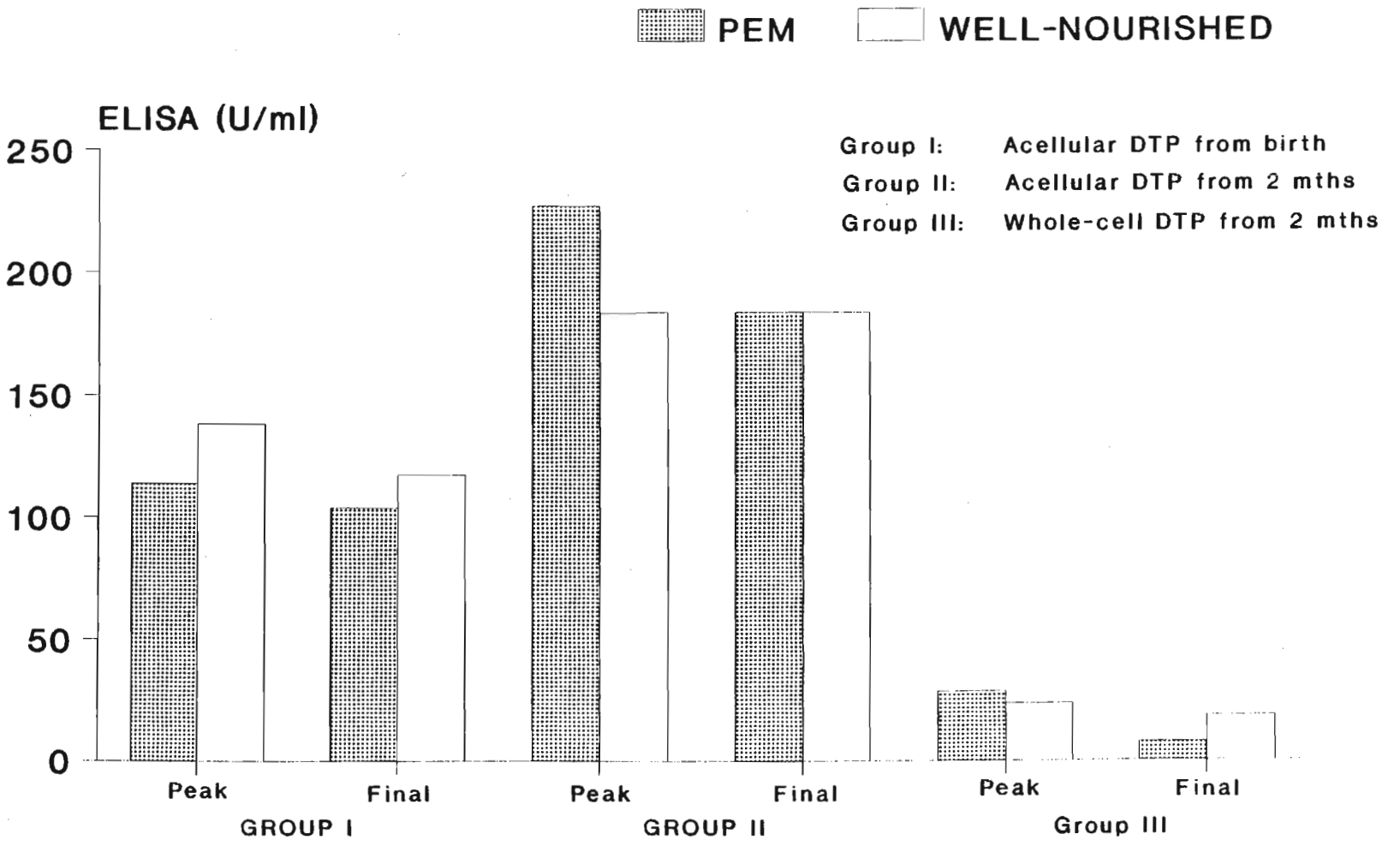


FIGURE 11.1 Peak and final IgG-anti-PT responses following acellular and whole-cell pertussis vaccine in PEM and well-nourished infants (Geometric Mean ± Standard Error).

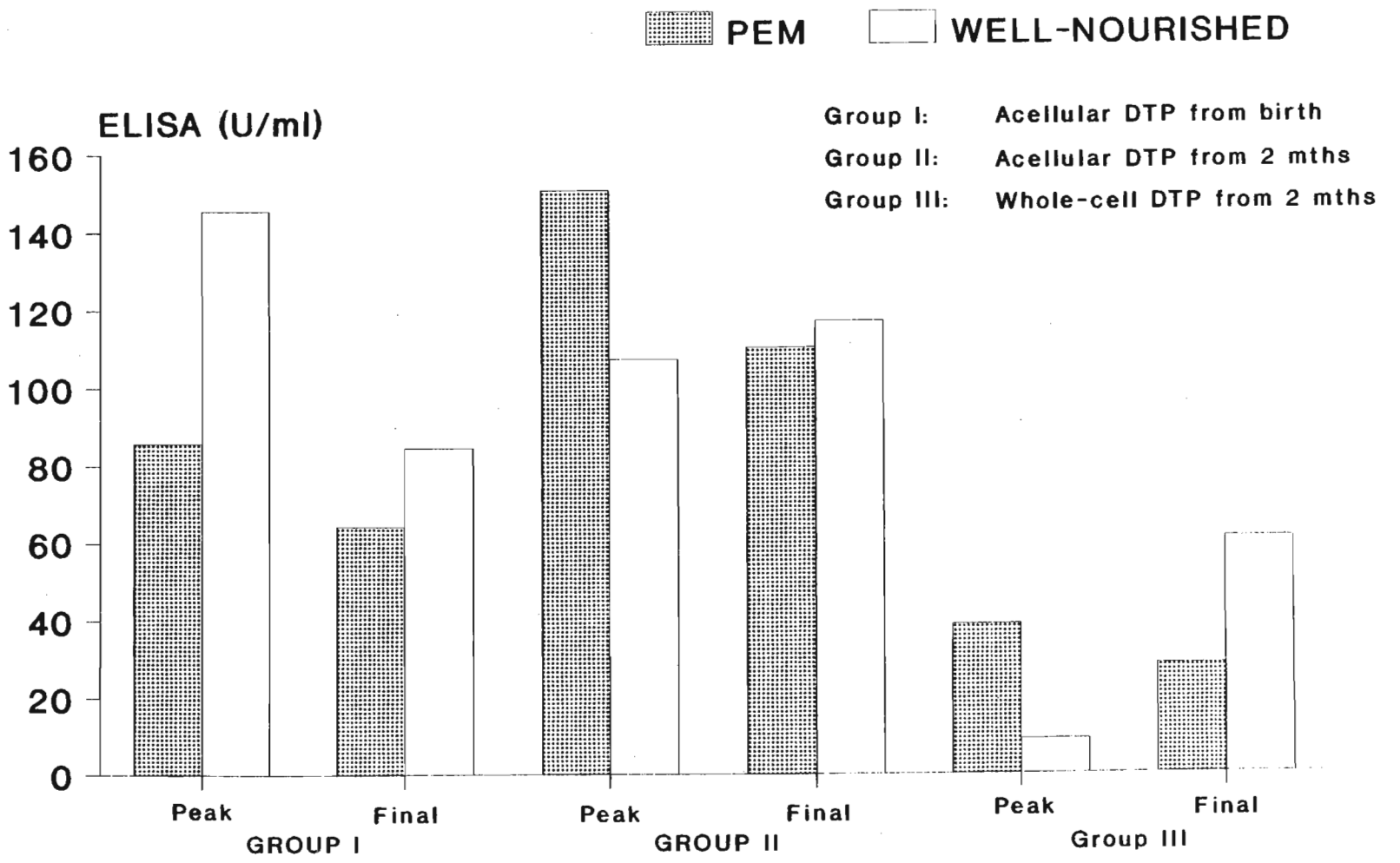


FIGURE 11.2 Peak and final IgG-anti-FHA responses following acellular and whole-cell pertussis vaccine in PEM and well-nourished infants (Geometric Mean ± Standard Error).

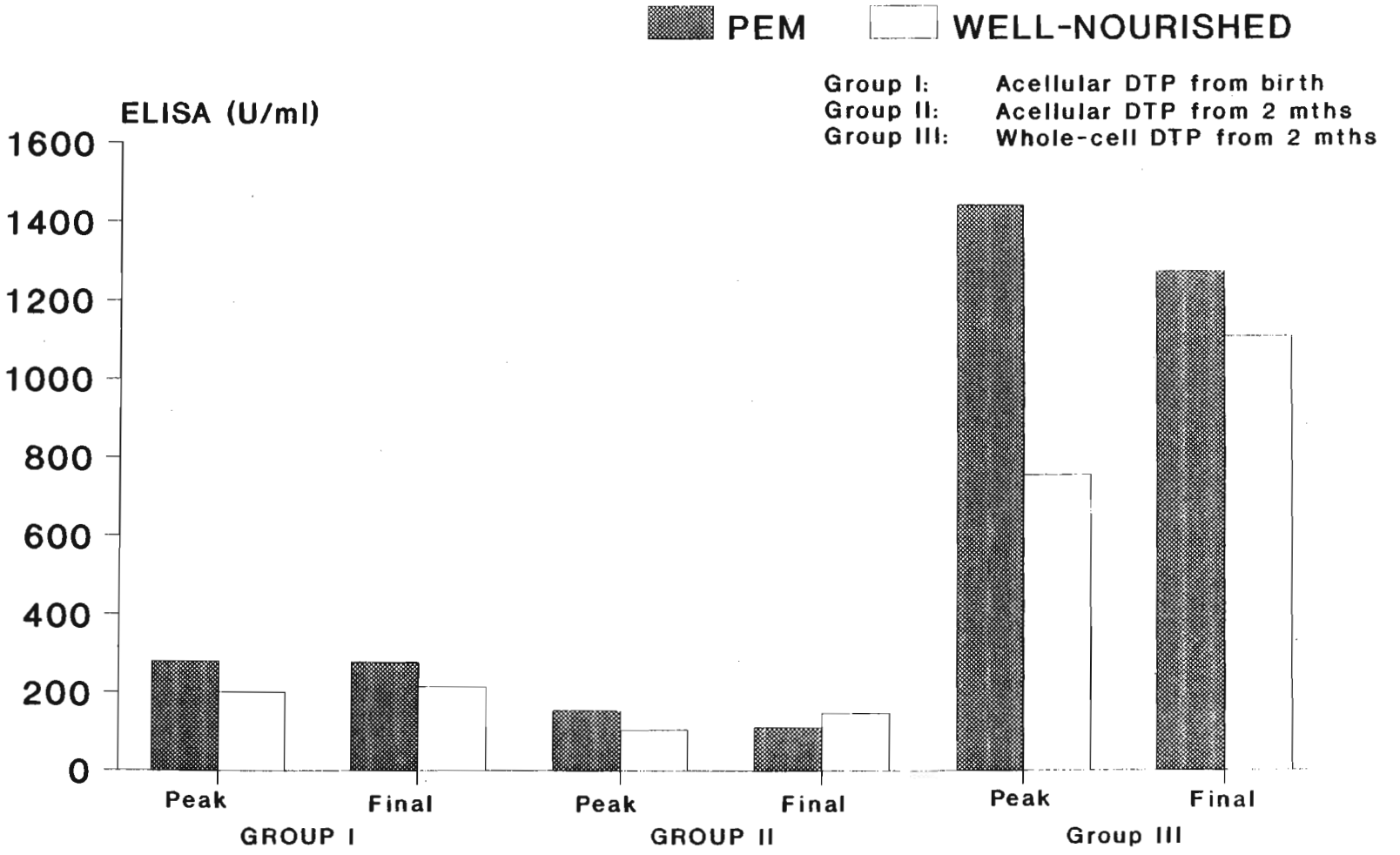


FIGURE 11.3 Peak and final IgG-anti-AGG2,3 responses following acellular and whole-cell pertussis vaccine in PEM and well-nourished infants (Geometric Mean ± Standard Error).

CHAPTER 12

QUALITY CONTROL STUDIES

12.1 PURPOSE OF STUDY

Study 1

To evaluate the nature and purity of pertussis reference antigen preparations by analytical SDS-PAGE.

Study 2

To evaluate the toxicity of acellular and whole-cell pertussis vaccines using Chinese hamster ovary (CHO) cells.

Study 3

To compare the antibody responses to pertussis vaccine measured by CHO neutralization, ELISA, and immunoblotting.

12.2 STUDY 1: EVALUATION OF REFERENCE PERTUSSIS ANTIGEN PREPARATION BY ANALYTICAL SDS-PAGE

INTRODUCTION

The nature and purity of the PT and FHA reference antigen preparation used in the detection of antibodies by ELISA and the neutralization assay were evaluated by analytical SDS-PAGE. These antigens do not migrate in gel buffer systems at basic pH values so a multiphasic acid was used.

A major concern in pertussis vaccine research is to produce a vaccine which contains the necessary protective antigens with a minimum content of toxic components. The reference antigen preparation evaluated in this assay was supplied by the Research Foundation for Microbial Diseases of Osaka University, Japan.

METHOD

Details of methods used are given in Chapter 5, hence only a brief account is given here. Analytical PAGE was done under reducing conditions. Gels were polymerized with ammonium persulphate. Gels were fixed and stained with 0.01% Coomassie blue R250.

RESULTS

Results of SDS gel electrophoresis of Japanese Reference antigen preparation containing PT and FHA is shown in Figure 12.1. The stained gel showed PT to be composed 1 major band of 5 subunit bands as major components with the following apparent molecular weights, 28, 23, 223, 11.7, 9.3 kDa. The FHA preparation showed 1 major band at 220 kDa, and a small amount of more slowly moving components.

DISCUSSION

SDS gel electrophoresis of other purified samples of PT have shown these proteins to be composed of 1 major band of 4 subunits with molecular weights between 27,000 and 12,000 (Arai & Sato, 1976; Irons & MacLennan, 1979; Morse & Morse, 1976) and FHA to be composed of 1 or 2 major subunits (MW 95-126,000). The reference pertussis antigen preparation was composed largely of purified PT and FHA.

12.3 STUDY 2: COMPARISON OF TOXICITY OF SOUTH AFRICAN WHOLE-CELL PERTUSSIS VACCINE AND JAPANESE (J-NIH-6) ACELLULAR PERTUSSIS VACCINE USING CHINESE HAMSTER OVARY (CHO) CELLS

INTRODUCTION

PT induces characteristic morphological changes in CHO cells and this phenomenon can be used to estimate the concentration of active PT present in test samples. CHO cells are extremely sensitive to minute traces of active toxin (detection of <10 ng PT/ml). The Centre for Applied Microbiology and Research (CAMR), UK reference PT material was used as a standard. The highest dilution of the toxin producing clustering of the cells was considered to represent

the toxin titre of the preparation.

MATERIALS AND METHODS

Details of materials and methods are given in Chapter 5.

CHO CELL CULTURE

A cell line maintained at the Centre for Applied Microbiological Research, Porton, UK by serial passages in Gibco Minimal Essential Medium (MEM) with 10% (v/v) newborn calf serum was used. Single cell suspensions were obtained from confluent monolayers of CHO cells by treatment with a solution of 0.1% (w/v) trypsin and 0.02% (w/v) EDTA in phosphate-buffered saline, pH 7.2. The released cells were adjusted to a final concentration of 40 $x10^3$ cells per ml MEM.

VACCINE PREPARATIONS

The acellular DTP vaccine used was the BIKEN B-type (J-NIH-6) adsorbed preparation from a single batch. One dose of acellular vaccine contained 28 Lf units of diphtheria toxoid, 7 Lf units of tetanus toxoid, 7.5 μg each of purified PT and FHA and 0.075 mg aluminium phosphate in phosphate-buffered saline (PBS) with 0.01% (w/v) thiomersal. The South African whole-cell DTP vaccine used was supplied as a single lot from the South African Institute for Medical Research, Johannesburg. One dose of whole-cell vaccine contained 25 Lf units of diphtheria toxoid, 6 Lf units of tetanus toxoid, 10,000 $x10^6$ *B. pertussis* organisms and 1.25 mg aluminium phosphate in PBS with 0.01% (w/v) thiomersal.

The clustering effect of PT on the CHO cells may be observed either directly with an inverted microscope or after staining.

The clustering of CHO cells is scored as :

0 = no clustering
1 = some clustering
2 = intermediate clustering
3 = 100% clustering

RESULTS

CHO assay: Comparison of vaccine toxicity.

TEST SAMPLE SCORE	RAW SCORE	MEAN RAW* SCORE	TOTAL DILUTION IN WELL (x)	CORRECTED DILUTION
CAMR PT standard	3.2; 3.2	2.5	50.9	786
J-NIH-6** (diluted ¼)	≤0; ≤0; ≤0	≤0	≤9x4 = ≤36	≤60.6
DTP (S. African) (diluted ¼)	≤1; ≤1; ≤1	≤1	≤18x4 = ≤72	≤121

* Related to toxin dilution

** Japanese acellular pertussis vaccine.

CALCULATION OF PT CONTENT

$$[PT] \text{ in test sample} = 40 \text{ ng/ml} \times \frac{1}{\text{pre-dilution}} \times 2^{(T-S)}$$

where,

T = mean raw score of test sample
S = mean raw score of standard.

hence,

J-NIH-6 $[PT] = 40 \text{ ng/ml} \times 2^{(\leq 0-2.5)} \times 4 = \leq 28.4 \text{ ng/ml}$
South African DTP $[PT] = 40 \text{ ng/ml} \times 2^{(\leq 1-2.5)} \times 4 = \leq 56.6 \text{ ng/ml}$

DISCUSSION

The South African whole-cell vaccine had approximately twice the PT (and hence toxicity) of the

acellular vaccine. The high level of toxicity in whole-cell vaccines is related to the wide range of side effects reported in vaccine recipients.

12.4 STUDY 3: COMPARISON OF ANTIBODY RESPONSES TO PERTUSSIS VACCINE MEASURED BY CHO, ELISA AND IMMUNOBLOTTING

INTRODUCTION

Pertussis toxin (PT) has been proposed to be the major cause of the harmful effect of prolonged immunity to pertussis (Pittman, 1979). Measurement of the antibody response to PT in natural disease and in vaccinees has been hampered by a lack of simple *in vitro* methods for such measurements.

An *in vitro* neutralization test in microplate cell culture (Gillenius *et al.*, 1985) based on the observation that PT induces a distinct CPE, ie. clustering in Chinese Hamster Ovary Cells was used to measure anti-PT antibodies in paired pre- and post-vaccination sera. ELISA and immunoblotting were performed using the same sera, and results compared.

PATIENTS AND METHODS

Details of patients and methods are provided in Chapters 4 and 5. Only a brief summary is presented here. The development of the anti-PT response was studied in paired serum samples obtained at birth and before vaccination at 2, 4, 6 and 9 months. The subjects whose antibody profiles are shown, received acellular pertussis vaccine from birth (subjects 1 and 2) or from 2 months of age (subject 3).

DETERMINATION OF ANTI-PT ANTIBODIES WITH CHO CELLS

The morphological changes in CHO cells induced by active PT as described in Chapter 5, can be inhibited by anti-PT antibodies. This phenomenon was used as the basis of the test estimating PT neutralising antibodies *in vitro*. Again, a reference serum with PT neutralising

activity was used.

DETERMINATION OF ANTI-PT ANTIBODIES BY ELISA

Microtitre plates coated with PT were used and the anti-PT activity of the test sera compared with those of the reference preparations.

IMMUNOBLOTTING

Immunoblotting was used to evaluate the specificity of the antibody response to candidate vaccines and to confirm the ELISA data. In immunoblotting, antigens were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose (NC) paper where they were allowed to react with antiserum. Antigen-antibody complexes were detected using an enzyme-substrate colour - development reaction. The presence of specific Ig's in sample sera was indicated by bands of *in situ* labelling of the relevant antigens on the NC paper. Each band is compared with controls scored as negative, weak positive or strongly positive.

CONTROL SERA

1. *Negative sera*: 20% newborn calf sera diluted in PBST.
2. *Weak positive sera:* Inactivated human serum non-reactive for pertussis antigens with low titres of antibody to pertussis antigens.
3. *Strongly positive sera:* Inactivated human sera non-reactive for pertussis antigens, with high titres of antibody to pertussis antigens.

STATISTICAL ANALYSIS

The mean titre and and fold increases were calculated on logarithmic values of titres determined by ELISA and by the neutralisation test and compared by the Student's t-test. The correlation between antibody titres obtained in the CHO assay and in ELISA were compared by regression analysis.

RESULTS

The anti-PT response measured by the CHO clumping neutralisation assay and by ELISA in 3 infants vaccinated with acellular pertussis vaccine is shown in Table 12.2 and Figure 12.2. The analysis of correlation between titres measured by ELISA and by CHO is shown in Figure 12.3. There was little or no relationship between values obtained by the 2 methods. Results of ELISA were confirmed by immunoblotting (data not given), the latter is however a qualitative assay.

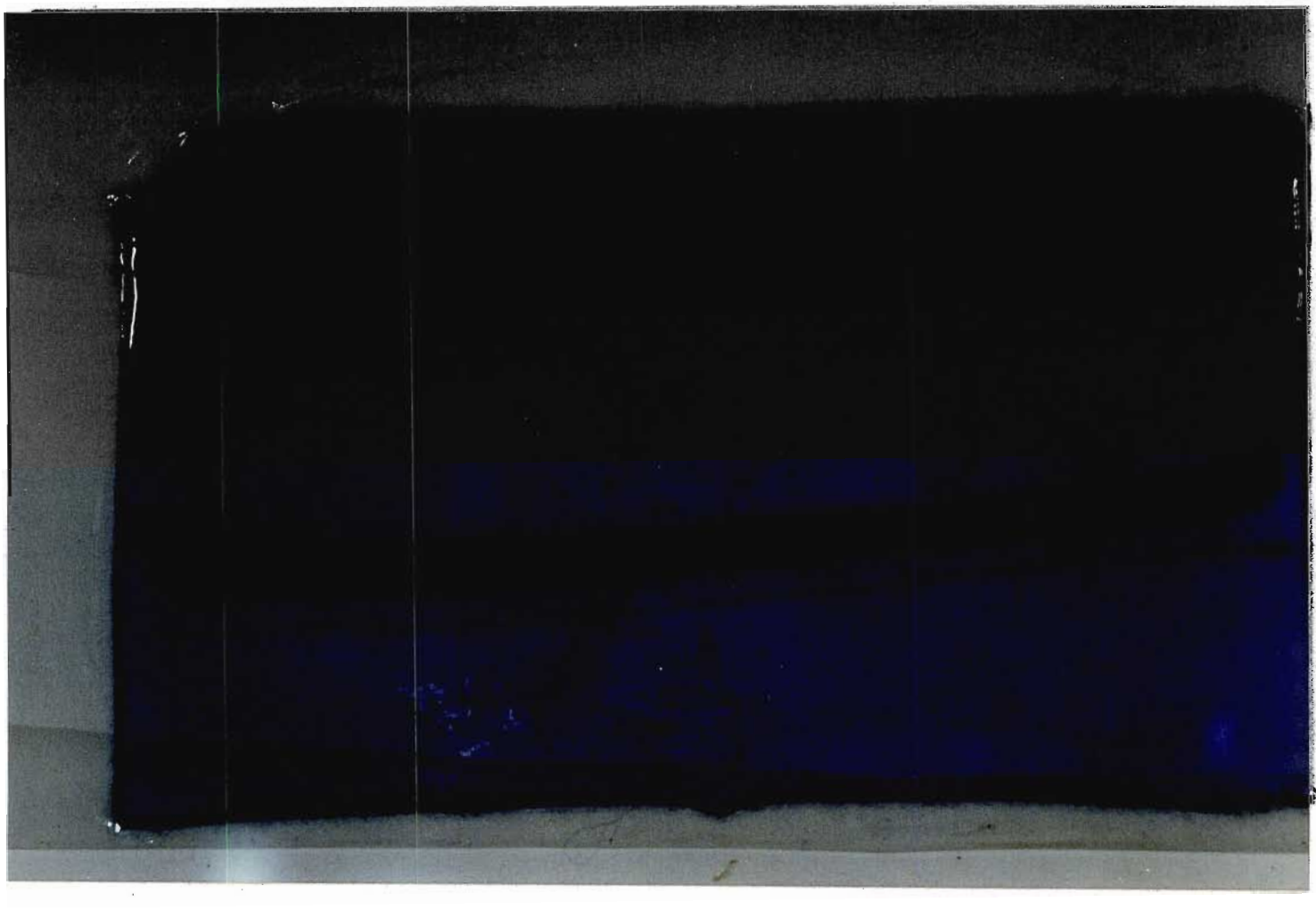
DISCUSSION

Zackrisson *et al.* (1990) and Trollfors *et al.* (1988) reported a lower sensitivity of the neutralisation test in comparison with ELISA; in accordance with the results obtained in this study. Immunoblotting is not suitable for the assay of pertussis antibodies when a quantitative evaluation is required.

TABLE 12.1 CHO inhibition: mean raw score related to antibody dilution.

SAMPLE NUMBER	RAW SCORE	MEAN RAW SCORE	ELISA (U/ml)
Plate 1			
Positive control	7 ; 7	7.0	
Maternal*	≤1 ; ≤1	≤1.0	1.15
Cord	1 ; 1	1.0	2.04
2 month	6 ; 5	5.5	58.97
4 month	7 ; ≤8	≥7.5	
6 month	≥8 ; ≥8	≥8.0	845.53
9 month	≥8 ; ≥8	≥8.0	186.46
Maternal*	3 ; 2	2.5	30.66
Cord	4 ; 4	4.0	26.25
2 month	2 ; 2	2.0	13.94
Plate 2			
Positive control	≥8 ; ≥8	≥8	
4 month	5 ; 4	4.5	50.71
6 month	4 ; 4	4.0	52.86
9 month	4 ; 4	4.0	260.49
Maternal*	≥8 ; ≥8	≥8.0	248.33
Cord	≥8 ; ≥8	≥8.0	201.46
2 month	6 ; 6	6.0	35.48
4 month	3 ; 3	3.0	25.18
6 month	5 ; 4	4.5	55.48
9 month	5 ; 5	5.0	41.73

* Samples from subjects 1, 2 and 3 respectively



Analytical PAGE was done with the pH4 gel system.
Gel was fixed and stained with Coomassie blue.

FIGURE 12.1 Polyacrylamide gel electrophoresis of reference antigen preparation.

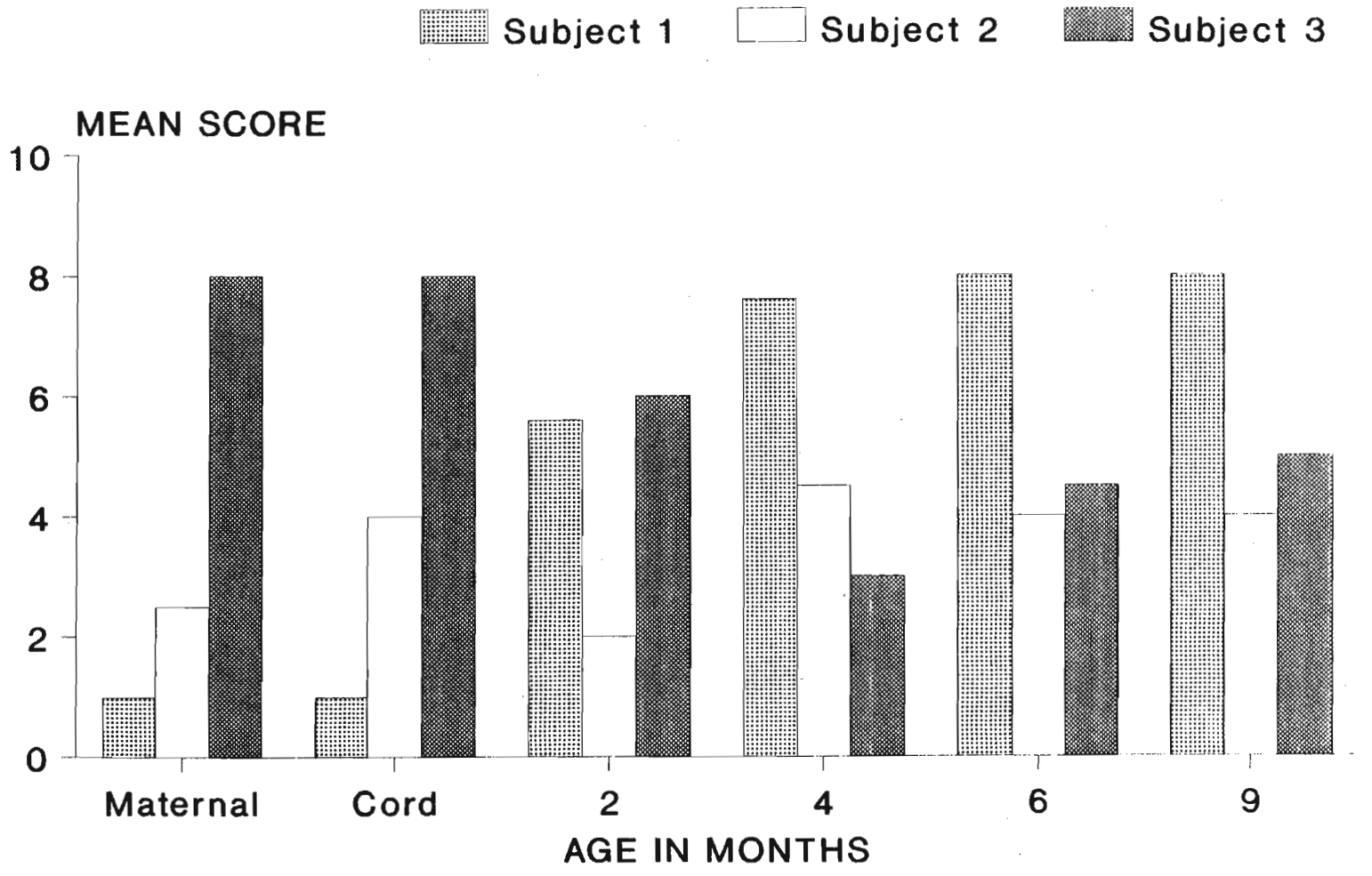


FIGURE 12.2 Antibody responses to pertussis vaccine as determined by neutralisation test.

Regression analysis - linear model: Y = a+bX

Dependent variable: CITITRE Independent variable: PT U/ml

Parameter	Estimate	Standard Error	T value	Prob Level
Intercept	1.44321	0.689246	2.09389	.06577
Slope	0.0664245	0.0173384	3.83107	.00402

Analysis of variance

Source	Sum of squares	Df	Mean square	F-ratio	Prob Level
Model	14.651846	1	14.651845	14.67709	.00402
Error	8.9845179	9	0.9982798		
Total (Corr)	23.636364	10			

Correlation coefficient = 0.787328 R-squared = 61.99 percent

Stnd Error of Est = 0.99914

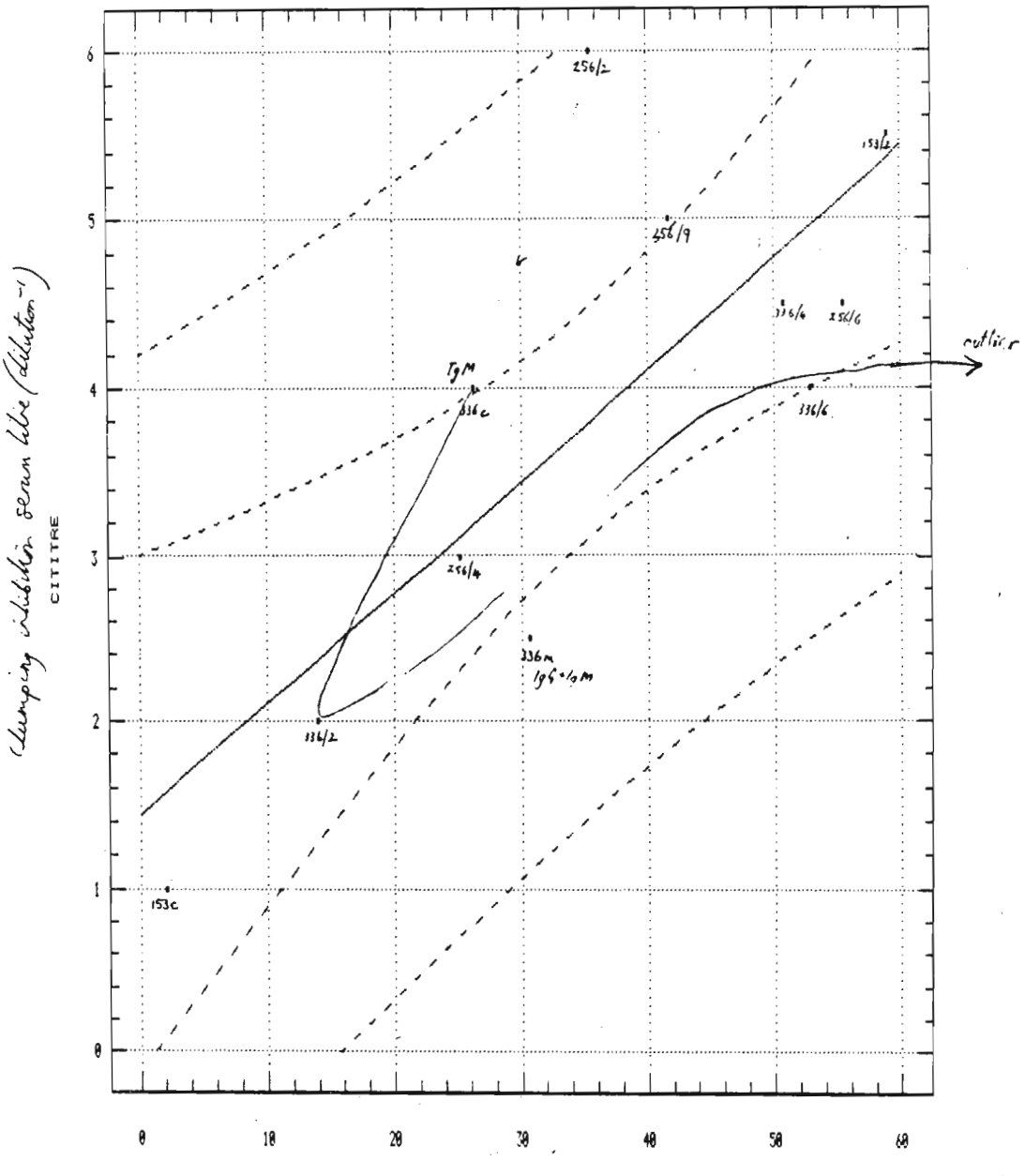


FIGURE 12.3 Analysis of correlation between antibody titres measured by ELISA and [illegible]

CONCLUSIONS AND RECOMMENDATIONS

Acellular and whole-cell pertussis vaccines were evaluated in South African infants. Two questions were addressed: whether the acellular vaccine would be safe and immunogenic if administered soon after birth; and whether it would prove more immunogenic and less toxic than the conventional vaccine when administered according to the routine schedule from 2 months of age.

The results of the study revealed that the South African whole-cell product was deficient both in reactogenicity and immunogenicity but that adequate responses were produced in recipients of acellular vaccine. Acellular vaccine was safest and produced the best antibody responses when incorporated into routine vaccination schedules; and whether given at birth or at 2 months, it proved safer and produced a better antibody response to PT and FHA than achieved with South African whole-cell vaccine. Overall, malnourished infants responded no less well to vaccination than did well-nourished infants.

Response to South African whole-cell vaccine was found merely to restore levels of serum IgG to PT and FHA to those found in cord blood. Serologic responses to PT and FHA, 2 principle antigens of *B. pertussis*, occurred infrequently and little or no increase in antibody titre was found after 3 doses of the vaccine. This study raises serious questions about the South African whooping cough vaccine which need to be resolved. Most importantly a wider study of vaccine safety and efficacy coupled with serological monitoring is urgently indicated to determine whether the results presented here are typical or if they arise from batch-to-batch variability of the vaccine. The poor immunogenicity of the whole-cell DTP used in this study, at least with

regard to FHA and PT, signifies that either (i) this batch would not provide protection, contrary to expectations based on reported efficacy; or (ii) any protection was likely to be due to some vaccine constituent(s) other than PT or FHA. At present the benefits of vaccination with the vaccine appears to outweigh the disadvantages, therefore despite the reservations expressed here, there is no indication for withdrawal of the vaccine and current vaccination programmes should be encouraged. Further, it is recommended that pertussis be made a notifiable disease in South Africa.

Subclinical pertussis was retrospectively diagnosed in 10 full-term and 3 pre-term infants on the basis of serologic evidence. Data from this study suggests that *B. pertussis* infection occurs in very young infants who are unvaccinated, incompletely vaccinated, or have had complete primary vaccination. This study extends the knowledge of contagious spread of subclinical infection to young infants with high levels of circulating maternally-acquired pertussis antibodies. Variable levels of PT, FHA and AGG2,3 IgG antibodies, ranging from low to high, were detected in maternal and cord sera. Suppressed clinical expression in these infants may have resulted from partial protection by maternally acquired (present in all) or vaccine-induced (present in some) pertussis antibodies. The presence of maternal antibodies is probably the end result of natural infection (as the currently used whole-cell vaccine produced poor responses to PT and FHA) and indicates that pertussis is widespread in this country. Nutritional status was not found to be an important factor in subclinical disease.

Although not designed to determine the protective effect of the vaccines used (sample size being too small to make a definite connection) the detection of subclinical pertussis infection in this study, highlights the limitations of clinical diagnostic criteria used in developing countries for disease surveillance and pertussis vaccine efficacy studies, as infection and transmission of the organism may occur in the absence of detectable clinical clues.

Considerably high levels of passively acquired pertussis, diphtheria and tetanus antibodies

were detected in pre-term infants. There was no discernible 'tolerance' due to neonatal DT vaccination, or to high levels of maternally acquired antibody in these infants. Although only preliminary, observations from this study suggest that the initiation of primary vaccination with DT at birth, followed by whole-cell DTP at 2, 4 and 6 months appears to be safe and effective in pre-term infants.

Although the size and design of the present trial prevents a definite conclusion, the results obtained imply that acellular vaccine can be given according to routine schedules to well nourished and poorly nourished, full-term and pre-term infants in developing countries, with the expectation of unrestricted antibody responses to the principle antigens and that the incidence of post-vaccination events will be no greater than that of conventional pertussis vaccine. These observations are particularly significant in the Third World where pertussis vaccines have to be given early in infancy since morbidity and mortality is highest in this age group. Further and more extensive efficacy studies are however indicated before the use of acellular pertussis vaccines can be recommended for routine primary vaccination of infants in preference to whole-cell preparations in developing countries.

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ACKNOWLEDGEMENTS

I wish to record my gratitude to Professor H.M. Coovadia who has been a source of constant inspiration and guidance to me throughout this study.

I am also deeply grateful to Professor W.E.K. Loening for his whole-hearted co-operation and helpful criticism at every stage of this study.

I acknowledge with gratitude the help of the Medical Research Council, South Africa; the British Council, and the Anglo-American-De Beers' Chairman's Fund, whose grants supported in part these investigations.

I wish to thank Dr. Iwao Yoshida, Research Foundation for Microbiological Diseases (BIKEN) Osaka, Japan for providing the acellular pertussis vaccine and City Health, Durban for providing the South African whole-cell pertussis and diphtheria-tetanus vaccines.

I would like to thank the Kwa Zulu Health Authorities for permitting the study of acellular and whole-cell pertussis vaccines to be undertaken at clinics under their jurisdiction.

I am grateful to Doctors G.L. Prosser, T.P. Gilpin, A.A. Freeman, M.A. Kibel, S. Knight, D.J. Power and L.G.V. Kustner for assistance in obtaining whooping cough data.

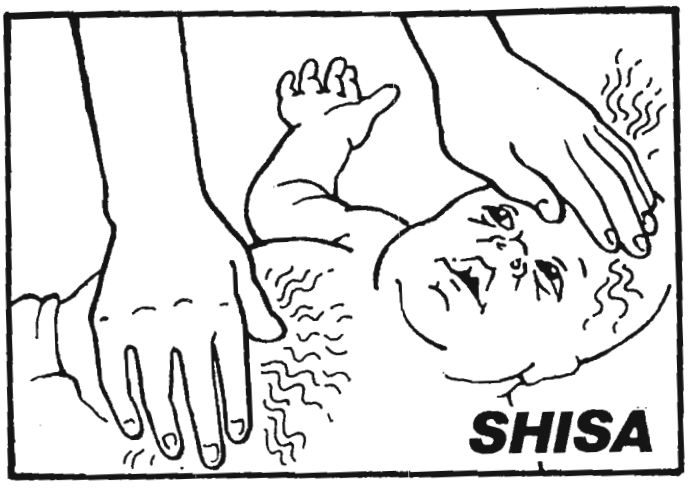
I wish to thank Dr. Elizabeth Miller and Dr. L.A.E. Ashworth of the PHLS, UK for their co-operation, use of laboratory facilities and erudite criticism.

My thanks are also due to Sister M. Ndlovana for her assistance with patient follow-up; Ms. Eleanor Gouws of the Institute of Biostatistics for analysis of data and Ms. Margaret Parkes and Ms. Eleanor Conner for typing of manuscripts.

I would like to express my appreciation to my husband Prakash and to my parents Dr. & Mrs. S. Ramkissoon for their encouragement and assistance at every stage.

APPENDIX 1

POST-VACCINATION EVENT RECORD SHEET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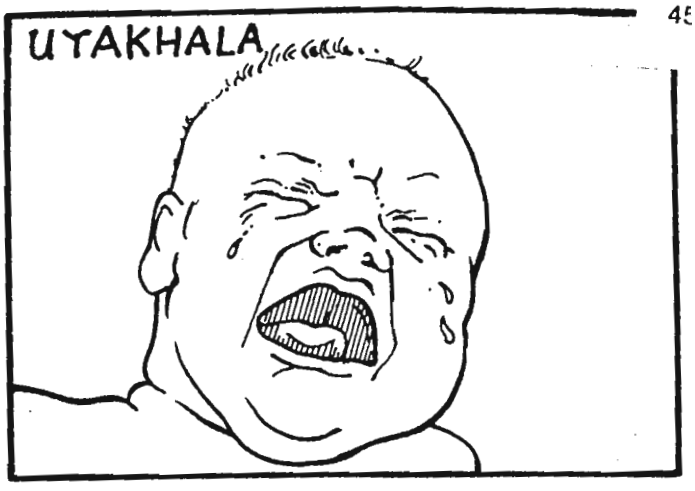
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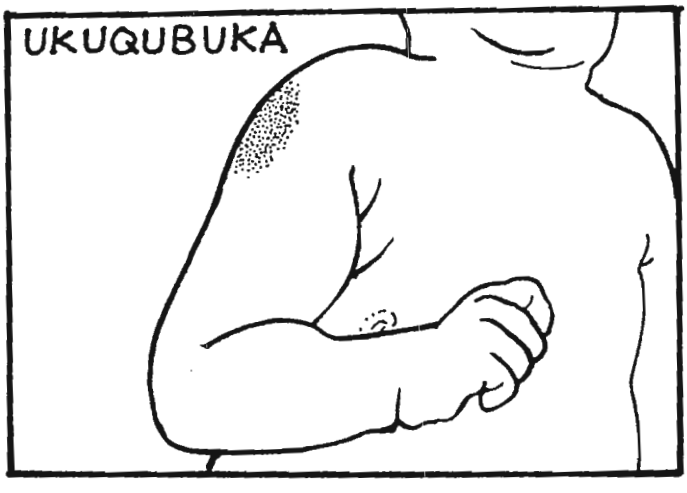
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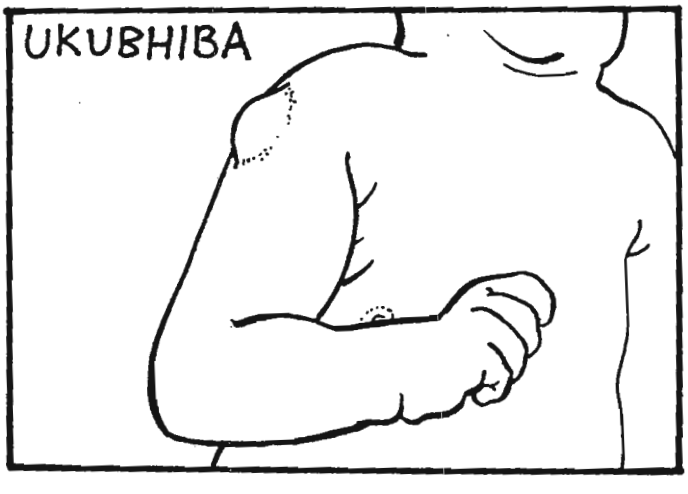
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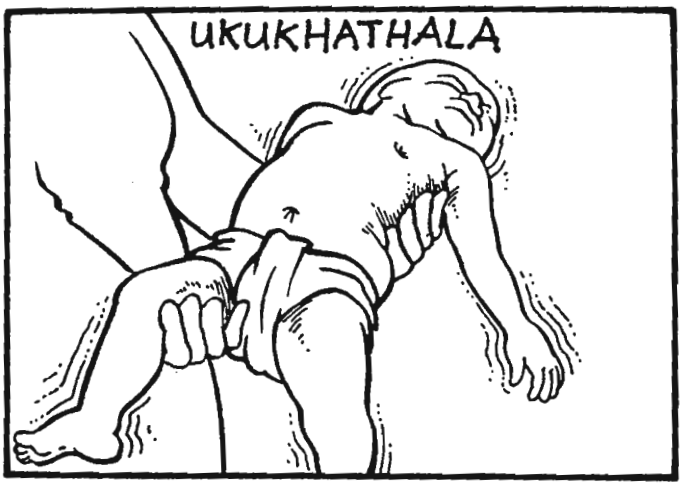
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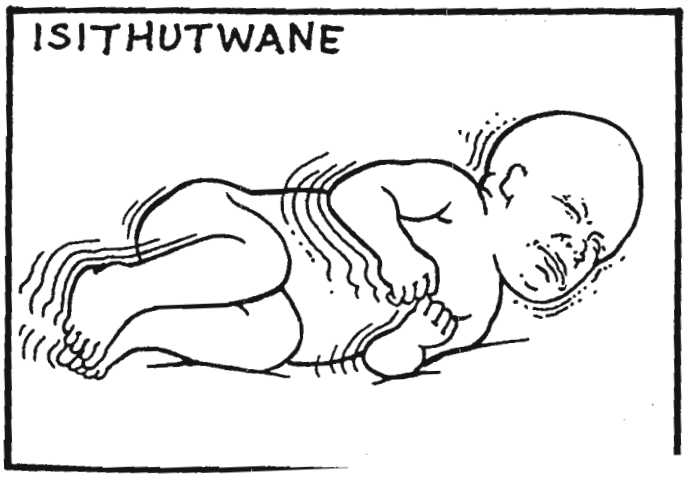
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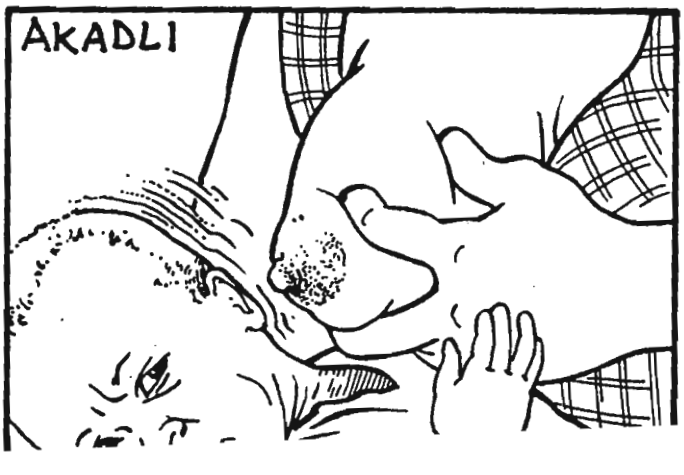
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APPENDIX 2

SUBJECT INFORMATION SHEET:
THE NEW WHOOPING COUGH VACCINATION

The clinic you will be attending for your baby's immunisation is one of the centres where a new vaccine against whooping cough is available and families are being invited to take part in a special study. This leaflet is to help you decide whether you would like your baby to be included.

A few years ago, scientists found a way of making purer whooping cough vaccines. These have been used successfully in Japan for the last 10 years. So far 40 million children have been immunised and these new style vaccines appear to give good protection with few side effects. Doctors in Sweden, France and America are already trying these vaccines and because of their encouraging reports many doctors feel that we should now be giving them to our own children.

At the moment, there are only limited supplies of the new types of vaccine here. These are being made available to clinics taking part in this special study which will compare the new vaccines with the present one. The results will help the Department of Health to choose the best vaccine for the immunisation programme in this country.

The new vaccines to be used in the study have already been successfully tested both in the laboratory and on adults. All the evidence suggests that they will be more effective and give even fewer side effects than the present vaccine.

What is Whooping Cough?

Whooping cough is an infectious disease that can be very serious for small babies. It is caused by a germ that attacks the air passages in the lung. A baby can be ill for months with bouts of coughing and choking followed by sickness. This leads to exhaustion and loss of weight. Some babies are permanently damaged and a few may die. Vaccination is the only way of protecting babies and young children.

How do we protect babies at the moment?

By an injection (immunisation) when the baby is 2 months old and repeated at about 4 and 6 months of age.

How are the new vaccines made?

The new vaccine is a purified version of the present one. Both are made from the germ that causes whooping cough, but the new one only contains those parts which are important for protection.

Are the new vaccines safe?

In Japan, where new vaccine have been used on a large scale for some years, they are accepted as safe. Reactions like a sore injection site or fever can occur but, so far, all the studies in other countries have shown that this is less likely with the new vaccines than the old ones.

How well do the new vaccines protect?

Studies done in Japan show that the new vaccines are effective in preventing children from catching whooping cough. In fact, they seem to protect young children whose brothers or sisters have whooping cough even better than the vaccine we now use in South Africa. However, it is sometimes difficult to compare the results of studies done in different countries and at different times. To be sure that the new vaccines are better than the ones we now use, they must be compared in the same study. This is why we are asking you to help us now.

What will be involved if my baby takes part in the study?

The majority of babies taking part in the study will be given one of the new whooping cough vaccines but some will be given the ordinary one for comparison. The study nurse will give you a record sheet to fill in. She will visit your home soon after vaccination and you can tell her how your baby has been. So that you are not influenced by knowing which vaccine was given, you will learn this only when the course is completed.

An essential part of the study will be to find out how well the vaccines have stimulated your baby's natural defenses against whooping cough. This can only be done by a blood test. For this the nurse will simply take a few drops from a heel or finger prick at each dose.

If you agree to take part, you will be asked to sign a consent form. A copy of this is enclosed with the leaflet. You would, of course, still be free to withdraw at any stage and to complete your baby's immunisation with the present vaccine. As with all studies of this kind, the Medical Research Council have ensured that all the usual safeguards have been followed and that the manufacturers accept full responsibility for their vaccines.

We do hope that your family will take part in this study. Your contribution would be an important step towards introducing a new whooping cough vaccine in South Africa and will help us to protect many more babies in the future.

APPENDIX 3

INVESTIGATOR CONTACT INFORMATION

IMPORTANT NOTICE

This child forms part of a vaccination study run by the Department of Paediatrics and Child Health, University of Natal.

This child has been vaccinated by us and we would like to supervise any further vaccination. If the child visits your hospital or clinic, for any reason whatsoever, would you be so kind as to contact one of the following:

Professor H.M. Coovadia	Telephone 2504347
Professor W.E.K. Loening	Telephone 2504352
Ms. A. Ramkissoon	Telephone 865122

KUBALULEKILE

Lengane ikhethiwe emsebenzini woku ncwaninga mayelana nokogoma izingane yi. Depatimenti yezingane yase yUnivesithi yase Natal.

Lengane igonywe yithine. Sifuna futhi ukulandela siyigome eminye elende layo. Uma lengane ilethiwe Kliniki noma esibhedlela sakso, siza uthintane namunye walaba:

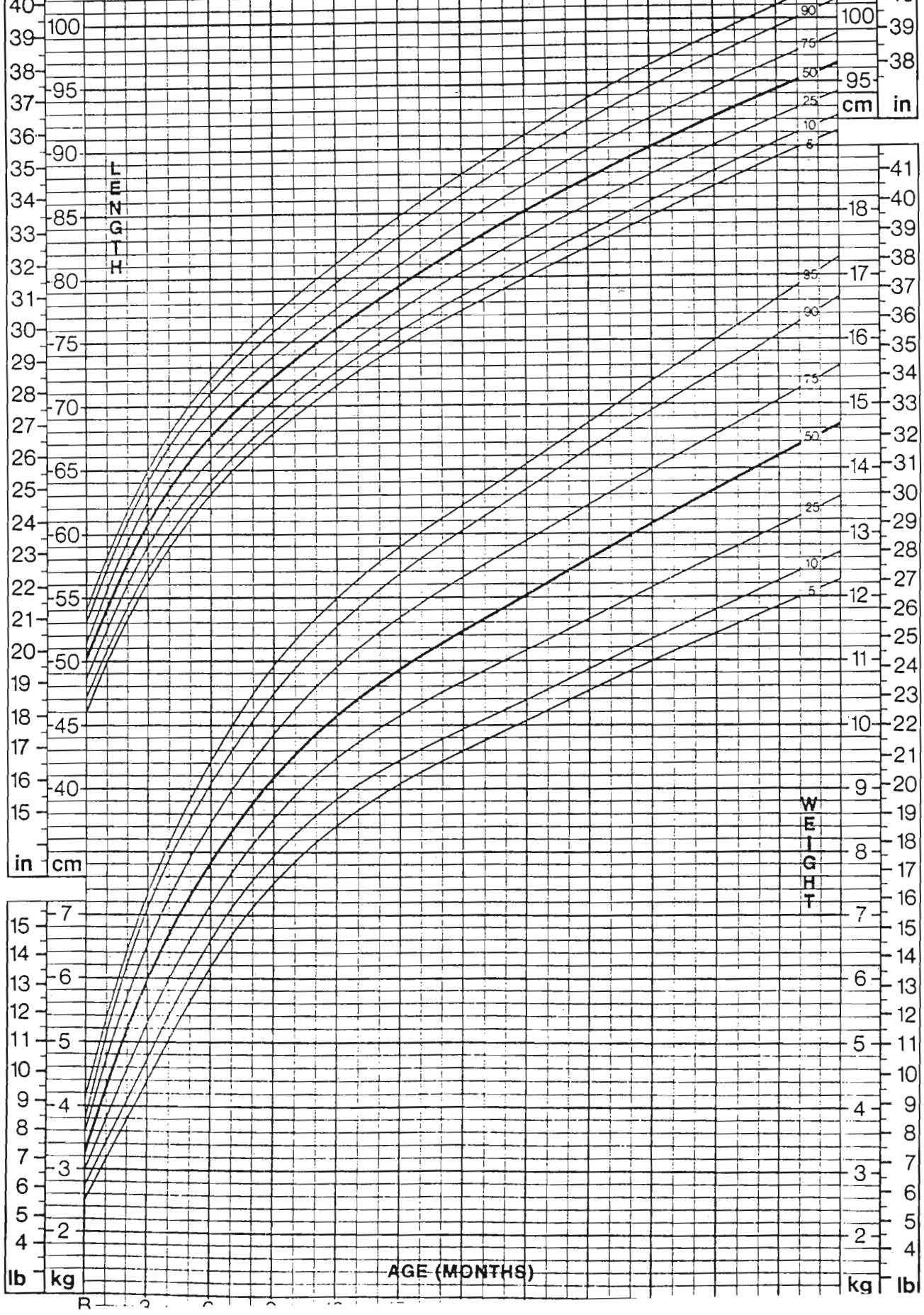
Professor H.M. Coovadia	Ucingo 2504347
Professor W.E.K. Loening	Ucingo 2504352
Ms. A. Ramkissoon	Ucingo 865122

APPENDIX 4

PHYSICAL GROWTH NCHS PERCENTILE GRAPHS FOR BOYS AND GIRLS

BOYS: BIRTH TO 36 MONTHS
PHYSICAL GROWTH
NCHS PERCENTILES*

NAME _____ RECORD # ____



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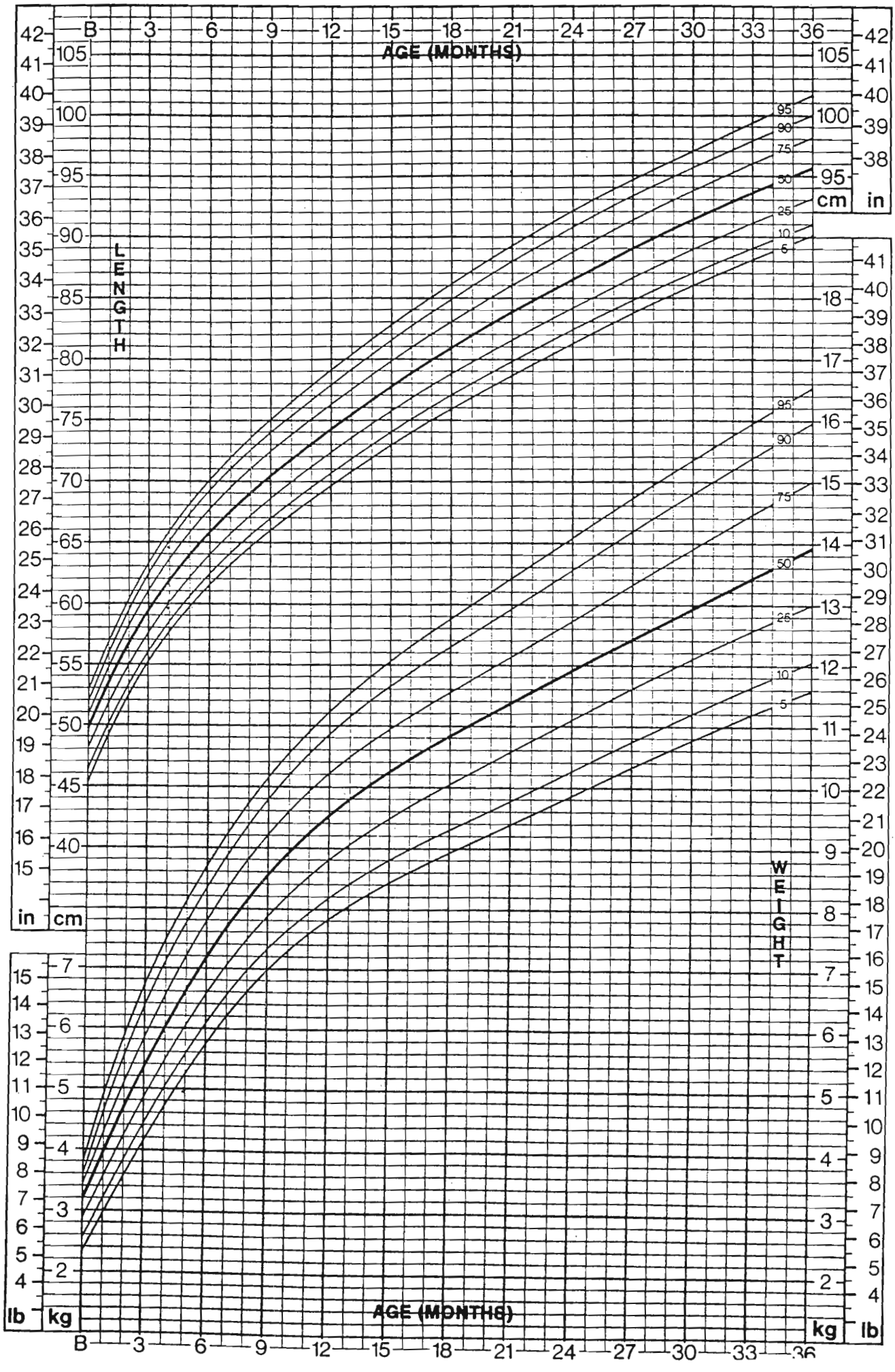
GIRLS: BIRTH TO 36 MONTHS
PHYSICAL GROWTH
NCHS PERCENTILES*

NAME _____ RECORD # _____



*Adapted from: Hamill PVV, Drizd TA, Johnson CL, Reed RB, Roche AF, Moore WM: Physical growth: National Center for Health Statistics percentiles. AM J CLIN NUTR 32:607-629 1979. Data from the Fels Research Institute, Wright

APPENDIX 5

NCHS PERCENTILE TABLES OF WEIGHT-FOR-AGE AND LENGTH-FOR-AGE INFANTS

Weight (kg) by Age of Boys aged 0–36 months

Age Months	Centiles													Standard deviations							Age Months
	3rd	5th	10th	20th	30th	40th	50th	60th	70th	80th	90th	95th	97th	−3S.D.	−2S.D.	−1S.D.	MEDIAN	+1S.D.	+2S.D.	+3S.D.	
0	2.5	2.6	2.7	2.9	3.1	3.2	3.3	3.4	3.5	3.7	3.9	4.1	4.2	2.0	2.4	2.9	3.3	3.8	4.3	4.8	0
1	3.0	3.2	3.4	3.7	3.9	4.1	4.3	4.5	4.6	4.9	5.1	5.4	5.6	2.2	2.9	3.6	4.3	5.0	5.6	6.3	1
2	3.6	3.8	4.1	4.5	4.7	5.0	5.2	5.4	5.6	5.9	6.2	6.5	6.7	2.6	3.5	4.3	5.2	6.0	6.8	7.6	2
3	4.2	4.4	4.8	5.2	5.5	5.7	6.0	6.2	6.4	6.7	7.1	7.4	7.6	3.1	4.1	5.0	6.8	6.9	7.7	8.6	3
4	4.8	5.1	5.4	5.8	6.2	6.4	6.7	6.9	7.2	7.5	7.9	8.2	8.4	3.7	4.7	5.7	6.7	7.6	8.5	9.4	4
5	5.4	5.7	6.0	6.5	6.8	7.0	7.3	7.5	7.8	8.1	8.5	8.9	9.1	4.3	5.3	6.3	7.3	8.2	9.2	10.1	5
6	6.0	6.2	6.6	7.0	7.3	7.6	7.8	8.1	8.4	8.7	9.1	9.4	9.7	4.9	5.9	6.9	7.8	8.8	9.8	10.8	6
7	6.5	6.7	7.1	7.5	7.8	8.1	8.3	8.6	8.9	9.2	9.6	10.0	10.2	5.4	6.4	7.4	8.3	9.3	10.3	11.3	7
8	7.0	7.2	7.5	8.0	8.3	8.5	8.8	9.0	9.3	9.6	10.1	10.5	10.7	5.9	6.9	7.8	8.8	9.8	10.8	11.8	8
9	7.4	7.6	7.9	8.4	8.7	8.9	9.2	9.4	9.7	10.1	10.5	10.9	11.1	6.3	7.2	8.2	9.2	10.2	11.3	12.3	9
10	7.7	7.9	8.3	8.7	9.0	9.3	9.5	9.8	10.1	10.4	10.9	11.3	11.5	6.6	7.6	8.6	9.5	10.6	11.7	12.7	10
11	8.0	8.2	8.6	9.0	9.3	9.6	9.9	10.1	10.4	10.8	11.3	11.6	11.9	6.9	7.9	8.9	9.9	10.9	12.0	13.1	11
12	8.2	8.5	8.8	9.3	9.6	9.9	10.2	10.4	10.7	11.1	11.6	12.0	12.2	7.1	8.1	9.1	10.2	11.3	12.4	13.5	12
13	8.5	8.7	9.1	9.5	9.9	10.1	10.4	10.7	11.0	11.4	11.9	12.3	12.5	7.3	8.3	9.4	10.4	11.5	12.7	13.8	13
14	8.7	8.9	9.3	9.8	10.1	10.4	10.7	10.9	11.3	11.6	12.1	12.6	12.8	7.5	8.5	9.6	10.7	11.8	13.0	14.1	14
15	8.8	9.1	9.5	10.0	10.3	10.6	10.9	11.2	11.5	11.9	12.4	12.8	13.1	7.6	8.7	9.8	10.9	12.0	13.2	14.4	15
16	9.0	9.2	9.6	10.1	10.5	10.8	11.1	11.4	11.7	12.1	12.6	13.0	13.3	7.7	8.8	10.0	11.1	12.3	13.5	14.7	16
17	9.1	9.4	9.8	10.3	10.7	11.0	11.3	11.6	11.9	12.3	12.8	13.3	13.6	7.8	9.0	10.1	11.3	12.5	13.7	14.9	17
18	9.3	9.5	10.0	10.5	10.9	11.2	11.5	11.8	12.1	12.5	13.0	13.5	13.8	7.9	9.1	10.3	11.5	12.7	13.9	15.2	18

Weight (kg) by Age of Girls aged 0–36 months

Age Months	Centiles													Standard deviations							Age Months
	3rd	5th	10th	20th	30th	40th	50th	60th	70th	80th	90th	95th	97th	−3S.D.	−2S.D.	−1S.D.	MEDIAN	+1S.D.	+2S.D.	+3S.D.	
0	2.3	2.4	2.6	2.8	3.0	3.1	3.2	3.3	3.4	3.5	3.7	3.8	3.9	1.8	2.2	2.7	3.2	3.6	4.0	4.3	0
1	2.9	3.0	3.2	3.5	3.7	3.8	4.0	4.1	4.3	4.4	4.7	4.9	5.0	2.2	2.8	3.4	4.0	4.5	5.1	5.6	1
2	3.4	3.6	3.8	4.1	4.4	4.5	4.7	4.9	5.1	5.3	5.6	5.8	6.0	2.7	3.3	4.0	4.7	5.4	6.1	6.7	2
3	4.0	4.2	4.4	4.8	5.0	5.2	5.4	5.6	5.8	6.1	6.4	6.7	6.9	3.2	3.9	4.7	5.4	6.2	7.0	7.7	3
4	4.6	4.7	5.0	5.4	5.6	5.8	6.0	6.3	6.5	6.8	7.1	7.4	7.6	3.7	4.5	5.3	6.0	6.9	7.7	8.6	4
5	5.1	5.3	5.6	6.0	6.2	6.4	6.7	6.9	7.1	7.4	7.8	8.1	8.3	4.1	5.0	5.8	6.7	7.5	8.4	9.3	5
6	5.6	5.8	6.1	6.5	6.8	7.0	7.2	7.4	7.7	8.0	8.4	8.7	8.9	4.6	5.5	6.3	7.2	8.1	9.0	10.0	6
7	6.0	6.2	6.5	6.9	7.2	7.5	7.7	7.9	8.2	8.5	8.9	9.3	9.5	5.0	5.9	6.8	7.7	8.7	9.6	10.5	7
8	6.4	6.6	7.0	7.4	7.7	7.9	8.2	8.4	8.7	9.0	9.4	9.8	10.0	5.3	6.3	7.2	8.2	9.1	10.1	11.1	8
9	6.7	7.0	7.3	7.7	8.1	8.3	8.6	8.8	9.1	9.4	9.8	10.2	10.4	5.7	6.6	7.6	8.6	9.6	10.5	11.5	9
10	7.0	7.3	7.6	8.1	8.4	8.7	8.9	9.2	9.4	9.8	10.2	10.6	10.8	5.9	6.9	7.9	8.9	9.9	10.9	11.9	10
11	7.3	7.6	7.9	8.4	8.7	9.0	9.2	9.5	9.8	10.1	10.6	10.9	11.2	6.2	7.2	8.2	9.2	10.3	11.3	12.3	11
12	7.6	7.8	8.2	8.6	9.0	9.3	9.5	9.8	10.1	10.4	10.9	11.2	11.5	6.4	7.4	8.5	9.5	10.6	11.6	12.7	12
13	7.8	8.0	8.4	8.9	9.2	9.5	9.8	10.1	10.3	10.7	11.1	11.5	11.8	6.6	7.6	8.7	9.8	10.8	11.9	13.0	13
14	8.0	8.2	8.6	9.1	9.5	9.8	10.0	10.3	10.6	10.9	11.4	11.8	12.0	6.7	7.8	8.9	10.0	11.1	12.2	13.2	14
15	8.1	8.4	8.8	9.3	9.7	10.0	10.2	10.5	10.8	11.2	11.6	12.0	12.3	6.9	8.0	9.1	10.2	11.3	12.4	13.5	15
16	8.3	8.6	9.0	9.5	9.9	10.2	10.4	10.7	11.0	11.4	11.9	12.3	12.5	7.0	8.2	9.3	10.4	11.5	12.6	13.7	16
17	8.5	8.7	9.2	9.7	10.0	10.3	10.6	10.9	11.2	11.6	12.1	12.5	12.7	7.2	8.3	9.5	10.6	11.8	12.9	14.0	17
18	8.6	8.9	9.3	9.8	10.2	10.5	10.8	11.1	11.4	11.8	12.3	12.7	13.0	7.3	8.5	9.7	10.8	12.0	13.1	14.2	18

Length (cm) by Age of Girls aged 0–36 months

Age Months	Centiles													Standard deviations							Age Months
	3rd	5th	10th	20th	30th	40th	50th	60th	70th	80th	90th	95th	97th	−3S.D.	−2S.D.	−1S.D.	MEDIAN	+1S.D.	+2S.D.	+3S.D.	
0	45.8	46.3	47.1	48.0	48.7	49.3	49.9	50.4	51.0	51.7	52.6	53.4	53.9	43.4	45.5	47.7	49.9	52.0	54.2	56.4	
1	49.2	49.8	50.6	51.6	52.3	53.0	53.5	54.1	54.8	55.5	56.5	57.3	57.9	46.7	49.0	51.2	53.5	55.8	58.1	60.4	1
2	52.2	52.8	53.7	54.7	55.5	56.1	56.8	57.4	58.0	58.8	59.8	60.7	61.3	49.6	52.0	54.4	56.8	59.2	61.6	64.0	2
3	54.9	55.5	56.4	57.5	58.2	58.9	59.5	60.2	60.9	61.6	62.7	63.6	64.2	52.1	54.6	57.8	59.5	62.0	64.5	67.0	3
4	57.2	57.8	58.7	59.8	60.6	61.3	62.0	62.6	63.3	64.1	65.2	66.2	66.8	54.3	56.9	59.4	62.0	64.5	67.1	69.6	4
5	59.2	59.8	60.7	61.9	62.7	63.4	64.1	64.7	65.4	66.3	67.4	68.4	69.0	56.3	58.9	61.5	64.1	66.7	69.3	71.9	5
6	61.0	61.6	62.5	63.7	64.5	65.3	65.9	66.6	67.3	68.2	69.3	70.3	70.9	58.0	60.6	63.3	65.9	68.6	71.2	73.9	6
7	62.5	63.2	64.1	65.3	66.2	66.9	67.6	68.3	69.0	69.8	71.0	72.0	72.6	59.5	62.2	64.9	67.6	70.2	72.9	75.6	7
8	64.0	64.6	65.6	66.8	67.6	68.4	69.1	69.7	70.5	71.3	72.5	73.5	74.2	60.9	63.7	66.4	69.1	71.8	74.5	77.2	8
9	65.3	66.0	66.9	68.1	69.0	69.8	70.4	71.1	71.9	72.8	74.0	74.9	75.6	62.2	65.0	67.7	70.4	73.2	75.9	78.7	9
10	66.6	67.2	68.2	69.5	70.3	71.1	71.8	72.5	73.2	74.1	75.3	76.3	77.0	63.5	66.2	69.0	71.8	74.5	77.3	80.1	10
11	67.8	68.5	69.5	70.7	71.6	72.4	73.1	73.8	74.5	75.4	76.6	77.7	78.3	64.7	67.5	70.3	73.1	75.9	78.7	81.5	11
12	69.0	69.6	70.7	71.9	72.8	73.6	74.3	75.0	75.8	76.7	77.9	79.0	79.6	65.8	68.6	71.5	74.3	77.1	80.0	82.8	12
13	70.1	70.8	71.8	73.1	74.0	74.8	75.5	76.2	77.0	77.9	79.2	80.2	80.9	66.9	69.8	72.6	75.5	78.4	81.2	84.1	13
14	71.2	71.9	72.9	74.2	75.1	75.9	76.7	77.4	78.2	79.1	80.4	81.4	82.1	67.9	70.8	73.7	76.7	79.6	82.5	85.4	14
15	72.2	72.9	74.0	75.3	76.2	77.0	77.8	78.5	79.3	80.3	81.6	82.6	83.3	68.9	71.9	74.8	77.8	80.7	83.7	86.6	15
16	73.2	73.9	75.0	76.3	77.3	78.1	78.9	79.6	80.4	81.4	82.7	83.8	84.5	69.9	72.9	75.9	78.9	81.8	84.8	87.8	16
17	74.2	74.9	76.0	77.4	78.3	79.1	79.9	80.7	81.5	82.5	83.8	84.9	85.6	70.8	73.8	76.9	79.9	82.9	86.0	89.0	17
18	75.1	75.9	77.0	78.3	79.3	80.1	80.9	81.7	82.5	83.5	84.9	86.0	86.7	71.7	74.8	77.9	80.9	84.0	87.1	90.1	18

Length (cm) by Age of Boys aged 0–36 months

Age Months	Centiles													Standard deviations							Age Months
	3rd	5th	10th	20th	30th	40th	50th	60th	70th	80th	90th	95th	97th	−3S.D.	−2S.D.	−1S.D.	MEDIAN	+1S.D.	+2S.D.	+3S.D.	
0	46.2	46.7	47.6	48.6	49.3	49.9	50.5	51.1	51.7	52.4	53.4	54.2	54.8	43.6	45.9	48.2	50.5	52.8	55.1	57.4	0
1	49.9	50.5	51.4	52.5	53.3	53.9	54.6	55.2	55.9	56.6	57.7	58.6	59.2	47.2	49.7	52.1	54.6	57.0	59.5	61.9	1
2	53.2	53.9	54.8	55.9	56.7	57.4	58.1	58.7	59.4	60.2	61.4	62.3	62.9	50.4	52.9	55.5	58.1	60.7	63.2	65.8	2
3	56.1	56.8	57.7	58.9	59.7	60.4	61.1	61.8	62.5	63.3	64.5	65.5	66.1	53.2	55.8	58.5	61.1	63.7	66.4	69.0	3
4	58.6	59.3	60.3	61.4	62.3	63.0	63.7	64.4	65.1	66.0	67.1	68.1	68.7	55.6	58.3	61.0	63.7	66.4	69.1	71.7	4
5	60.8	61.5	62.5	63.6	64.5	65.2	65.9	66.6	67.3	68.2	69.4	70.3	71.0	57.8	60.5	63.2	65.9	68.6	71.3	74.0	5
6	62.8	63.4	64.4	65.6	66.4	67.1	67.8	68.5	69.2	70.1	71.3	72.2	72.9	59.8	62.4	65.1	67.8	70.5	73.2	75.9	6
7	64.5	65.1	66.1	67.2	68.1	68.8	69.5	70.2	70.9	71.7	72.9	73.9	74.5	61.5	64.1	66.8	69.5	72.2	74.8	77.5	7
8	66.0	66.6	67.6	68.7	69.6	70.3	71.0	71.6	72.4	73.2	74.4	75.3	76.0	63.0	65.7	68.3	71.0	73.6	76.3	78.9	8
9	67.4	68.0	68.9	70.1	70.9	71.7	72.3	73.0	73.7	74.6	75.7	76.7	77.3	64.4	67.0	69.7	72.3	75.0	77.6	80.3	9
10	68.7	69.3	70.2	71.4	72.2	73.0	73.6	74.3	75.0	75.9	77.0	78.0	78.6	65.7	68.3	71.0	73.6	76.3	78.9	81.6	10
11	69.9	70.5	71.5	72.6	73.5	74.2	74.9	75.6	76.3	77.1	78.3	79.3	79.9	66.9	69.6	72.2	74.9	77.5	80.2	82.9	11
12	71.0	71.6	72.6	73.8	74.7	75.4	76.1	76.8	77.5	78.4	79.5	80.5	81.2	68.0	70.7	73.4	76.1	78.8	81.5	84.2	12
13	72.1	72.7	73.7	74.9	75.8	76.5	77.2	77.9	78.7	79.5	80.7	81.7	82.4	69.0	71.8	74.5	77.2	80.0	82.7	85.5	13
14	73.1	73.8	74.8	76.0	76.9	77.6	78.3	79.1	79.8	80.7	81.9	82.9	83.6	70.0	72.8	75.6	78.3	81.1	83.9	86.7	14
15	74.1	74.7	75.8	77.0	77.9	78.7	79.4	80.1	80.9	81.8	83.1	84.1	84.8	70.9	73.7	76.6	79.4	82.3	85.1	88.0	15
16	75.0	75.7	76.7	78.0	78.9	79.7	80.4	81.2	82.0	82.9	84.2	85.2	85.9	71.7	74.6	77.5	80.4	83.4	86.3	89.2	16
17	75.9	76.6	77.6	78.9	79.9	80.7	81.4	82.2	83.0	83.9	85.3	86.3	87.0	72.5	75.5	78.5	81.4	84.4	87.4	90.4	17
18	76.7	77.4	78.5	79.8	80.8	81.6	82.4	83.2	84.0	85.0	86.3	87.4	88.1	73.3	76.3	79.4	82.4	85.4	88.5	91.5	18

Weight (kg) by length of Boys 49–103 cm in height

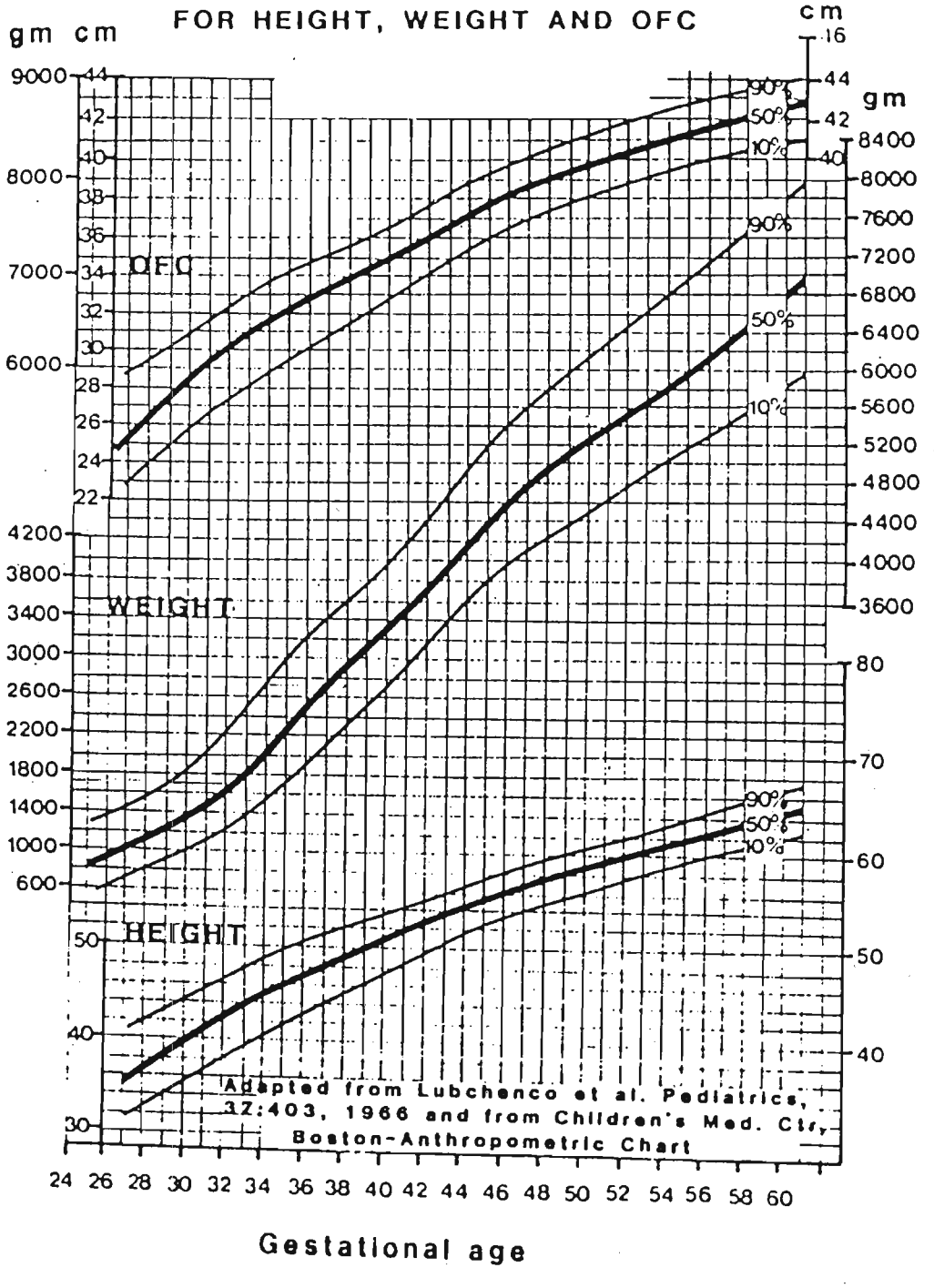
Length cm	Centiles													Standard deviations							Length cm
	3rd	5th	10th	20th	30th	40th	50th	60th	70th	80th	90th	95th	97th	−3S.D.	−2S.D.	−1S.D.	MEDIAN	+1S.D.	+2S.D.	+3S.D.	
49.0	2.5	2.6	2.7	2.9	3.0	3.1	3.1	3.3	3.4	3.6	3.8	4.0	4.1	2.1	2.5	2.8	3.1	3.7	4.2	4.7	49.0
49.5	2.5	2.6	2.8	2.9	3.0	3.1	3.2	3.4	3.5	3.7	3.9	4.1	4.2	2.1	2.5	2.9	3.2	3.7	4.3	4.8	49.5
50.0	2.6	2.7	2.8	3.0	3.1	3.2	3.3	3.4	3.6	3.7	4.0	4.2	4.3	2.2	2.5	2.9	3.3	3.8	4.4	4.9	50.0
50.5	2.6	2.7	2.9	3.1	3.2	3.3	3.4	3.5	3.7	3.8	4.1	4.3	4.4	2.2	2.6	3.0	3.4	3.9	4.5	5.0	50.5
51.0	2.7	2.8	2.9	3.1	3.3	3.4	3.5	3.6	3.8	3.9	4.2	4.4	4.5	2.2	2.6	3.1	3.5	4.0	4.6	5.1	51.0
51.5	2.8	2.9	3.0	3.2	3.3	3.5	3.6	3.7	3.9	4.0	4.3	4.5	4.6	2.3	2.7	3.1	3.6	4.1	4.7	5.2	51.5
52.0	2.8	2.9	3.1	3.3	3.4	3.6	3.7	3.8	4.0	4.1	4.4	4.6	4.7	2.3	2.8	3.2	3.7	4.2	4.8	5.4	52.0
52.5	2.9	3.0	3.2	3.4	3.5	3.7	3.8	3.9	4.1	4.3	4.5	4.7	4.9	2.4	2.8	3.3	3.8	4.3	4.9	5.5	52.5
53.0	3.0	3.1	3.3	3.5	3.6	3.8	3.9	4.0	4.2	4.4	4.6	4.8	5.0	2.4	2.9	3.4	3.9	4.5	5.0	5.6	53.0
53.5	3.0	3.2	3.3	3.6	3.7	3.9	4.0	4.1	4.3	4.5	4.7	5.0	5.1	2.5	3.0	3.5	4.0	4.6	5.2	5.8	53.5
54.0	3.1	3.3	3.4	3.7	3.8	4.0	4.1	4.3	4.4	4.6	4.9	5.1	5.2	2.6	3.1	3.6	4.1	4.7	5.3	5.9	54.0
54.5	3.2	3.3	3.5	3.8	3.9	4.1	4.2	4.4	4.5	4.7	5.0	5.2	5.4	2.6	3.2	3.7	4.2	4.8	5.4	6.0	54.5
55.0	3.3	3.4	3.6	3.9	4.1	4.2	4.3	4.5	4.7	4.9	5.1	5.4	5.5	2.7	3.3	3.8	4.3	5.0	5.6	6.2	55.0
55.5	3.4	3.5	3.7	4.0	4.2	4.3	4.5	4.6	4.8	5.0	5.3	5.5	5.6	2.8	3.3	3.9	4.5	5.1	5.7	6.3	55.5
56.0	3.5	3.7	3.9	4.1	4.3	4.4	4.6	4.7	4.9	5.1	5.4	5.6	5.8	2.9	3.5	4.0	4.6	5.2	5.9	6.5	56.0
56.5	3.6	3.8	4.0	4.2	4.4	4.6	4.7	4.9	5.0	5.3	5.5	5.8	5.9	3.0	3.6	4.1	4.7	5.4	6.0	6.6	56.5
57.0	3.7	3.9	4.1	4.3	4.5	4.7	4.8	5.0	5.2	5.4	5.7	5.9	6.1	3.1	3.7	4.3	4.8	5.5	6.1	6.8	57.0
57.5	3.8	4.0	4.2	4.5	4.7	4.8	5.0	5.1	5.3	5.5	5.8	6.1	6.2	3.2	3.8	4.4	5.0	5.6	6.3	7.0	57.5

Length cm	Centiles													Standard deviations							Length cm
	3rd	5th	10th	20th	30th	40th	50th	60th	70th	80th	90th	95th	97th	−3S.D.	−2S.D.	−1S.D.	MEDIAN	+1S.D.	+2S.D.	+3S.D.	
58.0	4.0	4.1	4.3	4.6	4.8	5.0	5.1	5.3	5.5	5.7	6.0	6.2	6.4	3.3	3.9	4.5	5.1	5.8	6.4	7.1	58.0
58.5	4.1	4.2	4.4	4.7	4.9	5.1	5.2	5.4	5.6	5.8	6.1	6.4	6.5	3.4	4.0	4.6	5.2	5.9	6.6	7.3	58.5
59.0	4.2	4.3	4.6	4.9	5.0	5.2	5.4	5.6	5.7	6.0	6.3	6.5	6.7	3.5	4.1	4.8	5.4	6.1	6.7	7.4	59.0
59.5	4.3	4.5	4.7	5.0	5.2	5.4	5.5	5.7	5.9	6.1	6.4	6.7	6.8	3.6	4.2	4.9	5.5	6.2	6.9	7.6	59.5
60.0	4.4	4.6	4.8	5.1	5.3	5.5	5.7	5.8	6.0	6.2	6.6	6.8	7.0	3.7	4.4	5.0	5.7	6.4	7.1	7.8	60.0
60.5	4.6	4.7	5.0	5.3	5.5	5.6	5.8	6.0	6.2	6.4	6.7	7.0	7.1	3.8	4.5	5.1	5.8	6.5	7.2	7.9	60.5
61.0	4.7	4.9	5.1	5.4	5.6	5.8	5.9	6.1	6.3	6.5	6.9	7.1	7.3	4.0	4.6	5.3	5.9	6.7	7.4	8.1	61.0
61.5	4.8	5.0	5.2	5.5	5.7	5.9	6.1	6.3	6.5	6.7	7.0	7.3	7.4	4.1	4.8	5.4	6.1	6.8	7.5	8.3	61.5
62.0	5.0	5.1	5.4	5.7	5.9	6.1	6.2	6.4	6.6	6.8	7.2	7.4	7.6	4.2	4.9	5.6	6.2	7.0	7.7	8.4	62.0
62.5	5.1	5.3	5.5	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.3	7.6	7.8	4.3	5.0	5.7	6.4	7.1	7.8	8.6	62.5
63.0	5.2	5.4	5.6	5.9	6.2	6.4	6.5	6.7	6.9	7.1	7.5	7.7	7.9	4.5	5.2	5.8	6.5	7.3	8.0	8.8	63.0
63.5	5.4	5.5	5.8	6.1	6.3	6.5	6.7	6.9	7.1	7.3	7.6	7.9	8.1	4.6	5.3	6.0	6.7	7.4	8.2	8.9	63.5
64.0	5.5	4.7	5.9	6.2	6.5	6.6	6.8	7.0	7.2	7.5	7.8	8.1	8.2	4.7	5.4	6.1	6.8	7.6	8.3	9.1	64.0
64.5	5.6	5.8	6.1	6.4	6.6	6.8	7.0	7.2	7.4	7.6	7.9	8.2	8.4	4.9	5.6	6.3	7.0	7.7	8.5	9.3	64.5
65.0	5.8	6.0	6.2	6.5	6.7	6.9	7.1	7.3	7.5	7.8	8.1	8.4	8.6	5.0	5.7	6.4	7.1	7.9	8.7	9.4	65.0
65.5	5.9	6.1	6.3	6.7	6.9	7.1	7.3	7.5	7.7	7.9	8.3	8.5	8.7	5.1	5.8	6.5	7.3	8.0	8.8	9.6	65.5
66.0	6.1	6.2	6.5	6.8	7.0	7.2	7.4	7.6	7.8	8.1	8.4	8.7	8.9	5.3	6.0	6.7	7.4	8.2	9.0	9.8	66.0
66.5	6.2	6.4	6.6	6.9	7.2	7.4	7.6	7.8	8.0	8.2	8.6	8.9	9.0	5.4	6.1	6.8	7.6	8.3	9.1	9.9	66.5

APPENDIX 6

COMBINED INTRAUTERINE-NEONATAL GROWTH CHART FOR HEIGHT, WEIGHT AND OFC

COMBINED INTRAUTERINE-NEONATAL GROWTH CHART FOR HEIGHT, WEIGHT AND OFC



APPENDIX 7

DOCUMENTATION ON PERTUSSIS-DIPHTHERIA-TETANUS COMBINED VACCINE, PURIFIED ADSORBED "BIKEN" (B-type)

Lot Number 21B

Administrative Office -

The Research Foundation for Microbial Diseases of Osaka University
3-1 Yamada-oka, Suita-city, Osaka, 565 Japan
Phone: Osaka (06) 877-5121
Cable: BIKEN-ZAIDAN, JAPAN

Manufacturing Facility -

Kanonji Institute

The Research Foundation for Microbial Diseases of Osaka University
2-9-41 Yahata-cho, Kanonji-city, Kagawa, 768 Japan
Phone: Kanonji (0875) 25-4171
Cable: BIKEN-KOJ KANONJI, JAPAN

Name and address of manufacturer

Name THE RESEARCH FOUNDATION FOR MICROBIAL DISEASES OF OSAKA UNIVERSITY

Address 3-1, Yamada-Oka, Suita, 565 JAPAN Phone : (06) 877-5121

Name of responsible person for production Takeo Konobe TAKEO KONOBE

Name of responsible person for control tests Iwao Yoshida IWAO YOSHIDA

Date of manufacture of final product Jan.16, 1987

Nature of final product Precipitated

Volume of manufacture 19,620ml

No. of containers in final lot for each filling volume 1 ml x 19620

Strain

(a) B . Pertussis	Tohama phase-1
(b) C . Diphtheria	Park-Williams No.8
(c) CI. Tetani	Harvard A-47

(Bulk materials)

a) Bulk material of Pertussis (No. of bulk material) PL-17

Control tests on bulk material	Date of Test	Result
1. Protein nitrogen test	Dec. 9,1986	39.8ugPN/ml
2. Staining test	Dec. 8,1986	Satisfactory
3. Sterility test	Dec.22,1986	Satisfactory
4. Inactivation test	Dec.15,1986	Satisfactory
5. Freedom from heat-labile toxin test	Dec.17,1986	Satisfactory
6. Mouse body weight-decreasing toxicity test	Dec.13,1986	1.0 BWDU/ml
7. Mouse leukocyte-increasing toxicity test	Dec.15,1986	0.41 LPUA/ml
8. Mouse histamine-sensitizing toxicity test	Dec.16,1986	0.10 HSU/ml
9. Pyrogen test	Jun.19,1986	Satisfactory

b) Bulk material of Diphtheria Toxoid.

(No. of bulk material) KD-83

c) Bulk material of Tetanus Toxoid

(No. of bulk material) KT-19

(Final bulk)

Control tests on final bulk

1. Thimerosal content test (Chemical test)	Jan.28,1987	0.00982 w/v%
(Biological test)	Jan.29,1987	0.00921 w/v%
2. Staining test	Jan.29, 1987	Satisfactory
3. Sterility test	Jan.12, 1987	Satisfactory
4. Freedom from abnormal toxicity test		
(GP-test)	Jan.29,1987	Satisfactory
(Mouse-test)	Jan.29,1987	Satisfactory
5. Mouse body weight-decreasing toxicity test	Jan.24,1987	5.2 BWDU/ml

(Freedom product)

Control tests on final product	Date of Test	Result
1. Protein nitrogen content	Jan.29,1987	22.8 ugPN/m
2. Hydrogen ion concentration test	Jan.28,1987	6.29
3. Aluminium content test	Jan.28,1987	0.150 mg/ml
4. Thimerosal content test (Chemical test)	Jan.28,1987	0.00941 w/v%
5. Formaldehyde content test	Jan.28,1987	0.00488 w/v%
6. Sterility test	Feb.12,1987	Satisfactory
7. Freedom from abnormal toxicity test	Jan.29,1987	Satisfactory
8. Mouse body weight-decreasing toxicity test	Jan.24,1987	5.9 BWDU/ml
9. Mouse leukocyte-increasing toxicity test	Jan.26,1987	0.21 LPU/ml
10. Mouse histamine-sensitizing toxicity test	Jan.27,1987	0.03 HSU/ml
11. Detoxification test of Diphtheria toxoid	Feb.18,1987	Satisfactory
12. Detoxification test of Tetanus toxoid	Feb.24,1987	Satisfactory
13. Potency test (Test for Pertussis)	Feb.20,1987	13.0 IU/dose
(Test for Diphtheria)	Mar.16,1987	44.0 IU/dose
(Test for Tetanus)	Mar.16,1987	51.2 IU/dose

Biological test of bulk material of Pertussis (PL-17) for D.P.T. Combined Vaccine,Purified,Adsorbed, "BIKEN"(B-type)Lot No. 21B

1. Mouse body weight decreasing toxicity

Test No. and Date of test	Sample	Difference of body weight (ave. 10 mice) 1 dil	2 dil	4 dil	16 dil	64 dil	BWDU/ml
352 Dec.13, 1986	Reference	-2.45	-1.89	-1.78	-0.56	0.30	115
	PL-17	0.65					1.0
	Saline	0.62					1.1
	Reference / Saline						115

Note : Minimum requirement in Japan (Less than 10 BWDU/ml)

Test for mouse body weight decreasing toxicity

1) Materials : The test sample and toxicity reference shall be used. The diluent for toxicity reference shall be used isotonic sodium chloride solution.

2) Test procedures : Toxicity reference shall be diluted as a suitable logarithmic serial dilutions. The test sample and each dillution of toxicity reference shall be injected intra-peritoneally into at least 10 mice of about 4 weeks of age at a dose of 0.5 ml. The body weight shall be recorded before and 16 hours after injection.

3) Criterion for judgment : The activity of mouse body weight-decreasing toxicity of the test sample shall be no higher than 10 BWDU per ml upon statistical comparison of the results.

2. Pyrogen test

Test No. and Date of test	Sample	Difference of rectum temp. between pre and post injection. Dilution of test material (30ugPN/ml) 1 dil.	dil.	Result
87-04 May, 6,1987	PL-17	0.2 C 0 C 0 C	NT	Satisfactory

Note : Minimum requirement in Japan
The test given General Testing Methods shall apply to the test sample prepared by diluting in isotonic sodium chloride solution to make the concentration of one-fiftieth that of the final bulk.

3. Endotoxin content (by LAL-test)

Test No.	Date of test	Sample	Test-1	Test-2	Test-3
		NT			

Biological test of D.P.T. Combined Vaccine, Purified, Adsorbed, "BIKEN" (B-ty
final bulk or final product of Lot No. 21B

1. Mouse body weight decreasing toxicity

Test No. and Date of test	Sample	Difference of body weight (ave. 10 mice) 1 dil	2 dil	4 dil	16 dil	64 dil	BWDU/ml
355 Jan.24, 1987	Reference	-2.11	-1.54	-1.53	0.27	0.31	115
	P21B	-0.17					5.9
	Saline	0.73					1.4
	Reference						115
	Saline						
	Reference						115
	Saline						
Average	Reference						115
	Saline						

Note : Minimum requirement in Japan (Less than 10 BWDU/ml)

2. Pyrogen test

Test No. and Date of test	Sample	Difference of rectum temp. between pre and post injection. Dilution of test material 1 dil.	5 dil.	Result
87-04 May. 6, 1987	21B	0 0 0.3	NT	Satisfactory

3. Endotoxin content (by LAL-test)

Test No.	Date of test	Sample	Test-1	Test-2	Test-3
217	Aug.24, 1987	21B	3.01 ng/ml	NT	NT

4. Mouse leukocyte increasing toxicity

Test No. and Date of test	Sample	Number of WBC/mm3 (ave. of 10 mice)				Ratio	LPU/ml
		1 dil.	2 dil.	4 dil.	16 dil.		
355 Jan.24, 1987	Reference	50,120	33,880	24,550	16,980	4.79	5.0
	21B	13,800				1.32	0.21
	Saline	10,470				1.00	0.11
	Reference						
	Saline						
	Reference						
	Saline						
Average	Reference						
	Saline						

Note : Minimum requirement in Japan (Less than 0.5 LPU/ml)

Test for mouse leukocyte increasing toxicity

1) Materials : The test sample and toxicity reference shall be used. The diluent for toxicity reference shall be isotonic sodium chloride solution.

2) Test procedures : Toxicity reference shall be dilute as a suitable logarithmic serial dilutions. The test sample and each dilution of toxicity refernce shall be injected intra-peritoneally into at least 10 mice of about 4 weeks of age at a dose of 0.5 ml. The leukocyte counting on peripheral blood shall be counted 3 days after injection.

3) Criterion for judgment : The activity of mouse leukocyte-increasing toxicity of the test sample shall be no higher than 0.5 LPU per ml upon statistical comparison of the results.

5. Mouse histamine sensitizing toxicity

Test No. and Date of test	Sample	Rectum temp.(C) after 30 min. histamine injec.					HSU/ml
		1 dil.	2 dil.	4 dil.	16 dil.	64 dil.	
355	Reference	Temp. 30.80	31.23	31.06	32.40	34.91	5.0
		Death 10	10	8	4	0	
	21B	Temp. 35.32					0.03
Jan.27, 1987		Death 0					
	Sample	Temp. 36.68					0.01
		Death 0					
	Reference	Temp.					5.0
		Death					
		Temp.					
		Death					
	Saline	Temp.					
		Death					
	Reference	Temp.					5.0
		Death					
		Temp.					
		Death					
	Saline	Temp.					
		Death					
	Reference	Temp.					5.0
		Death					
		Temp.					
		Death					
	Saline	Temp.					
		Death					

Note : Minimum requirement in Japan (Less than 0.8 HSU/ml)

Test for mouse histamie sensitizing toxicity

1) Materials : The test sample, toxicity reference, and histamine dihydrochloride shall be used. The toxicity reference and histamine dihydrochloride shall be diluted in isotonic sodium chloride solution.

2) Test procedures : Toxicity reference shall be diluted as a suitable logarithmic serial dilutions. The test sample and each dilution of toxicity reference shall be injected intra-peritoneally into at least 10 mice of about 4 weeks of age at a dose of 0.5 ml. Four days after injection, 4 mg of histamine dihydrochloride shall be inoculated into each mouse. The rectum temperature sahll be recorded 30 minutes after histamine injection.

3) Criterion for judgment : The activity of mouse histamine-sensitizing toxicity of the test sample shall be no higher than 0.8 HSU per ml upon statistical comparison of the results.

6. Chemical test of D.P.T. Combined Vaccine,Purified,Adsorbed, "BIKEN" (B-type) Lot 21B

	Final bulk		Final product	
	Date of test	Result	Date of test	Result
Test for protein nitrogen content (ugPN/ml)		NT	Jan.29, 1987	23.1
Test for hydrogen ion concentration	Jan.28, 1987	6.25(18.4 C)	Jan.28, 1987	6.29 (18.7 C)
Test for thimerosal content (W/V %)	Jan.28, 1987	0.00982	Jan.28, 1987	0.00941
Test for aluminium content (mg/ml)		NT	Jan.29, 1987	0.150
Test for formaldehyde content (W/V%)		NT	Jan.28, 1987	0.00488
Osmotic pressure (mOsm/kg)	Jan.28, 1987	288	Jan.28, 1987	289

7. Sterility test

Final bulk		Final product	
Date of test	Result	Date of test	Result
Feb.12, 1987	Satisfactory	Feb.12, 1987	Satisfactory

8. Freedom from abnormal toxicity test

A. Final bulk(21B)

1. GP-test

GP strain :Hartley Sex of GP :female Dose/GP : 5.0 ml ip-injection
Date of start of test :Jan.22, 1987 Date of end of test :Jan .29, 1987

GP No.	Sample	Body weight before injection	Survive or dead on 7 days after injection	Body weight on 7 days after injection	Yes or No
1	Final bulk	344 g	Survive	369 g	Yes
2	Final bulk	357 g	Survive	369 g	Yes
3	Final bulk	358 g	Survive	366 g	Yes

2. Mouse test

Mouse strain : dd Y Sex of mouse : Male Dose/Mouse : 0.5 ml ip-injection
Date of start of test :Jan.22, 1987 Date of end of test :Jan.29, 1987

Mouse No.	Sample	Body weight before injection	Survive or dead on 7 days after injection	Body weight on 7 days after injection	Yes or No
1	Final bulk	24.2 g	Survive	29.0 g	Yes
2	Final bulk	23.0 g	Survive	27.0 g	Yes
3	Final bulk	26.1 g	Survive	30.2 g	Yes
4	Final bulk	24.3 g	Survive	29.5 g	Yes

B. Final product : 21B

GP strain :Hartley Sex of GP :female Dose/GP : 5.0 ml ip-injection
Date of start of test :Jan.22, 1987 Date of end of test :Jan.29, 1987

GP. No.	Sample	Body weight before injection	Survive or dead on 7 days after injection	Body weight on 7 days after injection	Yes or No
1	Final Product	335 g	Survive	373 g	Yes
2	Final Product	371 g	Survive	409 g	Yes
3	Final Product	376 g	Survive	405 g	Yes

9. Potency test of D.P.T. Combined Vaccine,Purified Adsorbed, "BIKEN"(B-type)

Lot No. 21B

A.Potency of Pertussis

Date of start of test : Jan.15, 1987 Date of end of test : Feb.20, 1987

i) Mouse strain : dd Y
ii) Mouse age : 4 weeks
iii) Weight and sex of mice : 20 g ± 2 g , female

iv) Number of mice per dose of vaccine : 16 mice
v) Date of immunization : Jan.15, 1987
vi) Challenge dose : 204 LD_{50}
vii) Date of challenge : Feb. 6, 1987
viii) Date of judgment : Feb.20, 1987

vx) Result of challenge test

	Dose/mouse (dilution)	Number of mice	survival	Death	ED50	Potency IU/ml	95% Confidence limits
	1/8	20	13	7			
Reference	1/40	20	5	15	0.0353ml	8.0	76-131
Lot L-29	1/200	20	0	20			
	1/8	20	15	5			
Test Vac.	1/40	20	12	8	0.0127ml	26.0	78-128
Lot 21B	1/200	20	3	17			

Challenge strain	Number of bac./mouse	Number of mice	Survival	Death	LD50	Living ratio of challenge bacteria
	500	10	2	8		
18-323	100	10	6	4	147	29.4 %
	20	10	9	1		

Potency of test vaccine 13.0 IU per single human dose.
95 % confidence limits of potency 5.4 - 31.2 IU per single human dose.

Note : 1) Minimum requirement in Japan (More than 4 IU per single human dose)

2) The potency shall be determined in mice by the intracerebral challenge method.

B. Potency of Diphtheria

Species of animals	Guinea-pig
Weight of animals	300-400 g
No. of animals per dose of toxoid	10
Date of immunization and volume of dilutions administered	Feb. 5, 1987 2 ml
Date of bleeding	Mar. 5, 1987
Date of end of test	Mar.16, 1987

Results of antitoxin titration

Toxoid	Dilution	Average of antitoxin titer
Reference toxoid Lot No.1 (68 IU/ml)	50	0.043
	100	0.014
	200	0.009
Test toxoid	70	0.0034
	140	0.0023
	280	0.0100

Potency of test toxoid 44 IU per single human dose
95% confidence limits of potency 28.75-66.25 IU per single human dose

Note : 1)Minimum requirement in Japan (More than 23.5IU per sirgle human dose)
2)The potency shall be determined in guinea pigs by the toxin challenge method or by the antitoxin titration method.

C. Potency of Tetanus

Species of animals	Guinea-pig
Weight of animals	300-400 g
No. of animals per dose of toxoid	10
Date of immunization and volume of dilutions administered	Feb. 5, 1987
Date of challenge	Mar. 9, 1987
Challenge dose	50LD50
Date of end of test	Mar.16, 1987

Result of challenge test

Toxoid	Dilution	Average of score
Refernce toxoid Lot No. 1 (64 IU/ml)	50	3.8
	100	3.2
	200	0.8
Test toxoid	70	4.0
	140	3.2
	280	1.4

Potency of test toxoid 51.2 IU per single human dose

95% congidence limits of potency 38.75 - 68.8 IU per single human dose

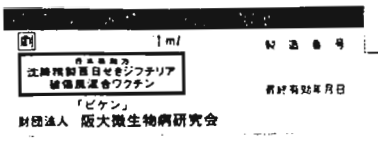
Note : 1) Minimum requirement in Japan (More than 13.5 IU per single human dose)

2) The potency shall be determined in guinea pigs by the toxin challenge method or by the antitoxin titretion method

APPENDIX 8

PERTUSSIS-DIPHTHERIA-TETANUS VACCINE, PURIFIED, ADSORBED "BIKEN"

PERTUSSIS-DIPHTHERIA-TETANUS COMBINED VACCINE, PURIFIED, ADSORBED "BIKEN"



[DESCRIPTION] This is a liquid preparation rendered insoluble by addition of aluminium salt into solution containing defensive antigen of pertussis bacilli and into solution containing diphtheria toxoid and tetanus toxoid. When shaken, it forms a uniform white turbidity.

pH: 5.4 - 7.4

Osmotic pressure ratio (to physiological saline solution): Approximately 1

[OUTLINE OF METHOD OF MANUFACTURE] The preparation contains a mixture, at fixed concentrations, of infection-defensive antigen fraction obtained by purifying cultured filtrate of pertussis I-phase bacilli (Higashihama strain) by salting-out and density-gradient ultra-centrifugation of sucrose and then inactivating and detoxifying with formalin, of diphtheria toxoid obtained by purifying the toxin in cultured filtrate of dephtheria bacilli (Park-Williams No. 8 strain) by salting-out and then detoxifying it with formalin, and of tetanus toxoid obtained by purifying the toxin in cultured filtrate of tetanus bacilli (Harvard strain), and it is a liquid preparation rendered insoluble through adsorption with aluminium phosphate to raise its immunogenicity.

Each ml of the preparation contains defensive antigen of pertussis bacilli at 8IU or more, about 30Lf of the diphtheria toxoid and about 5Lf of tetanus toxoid.

The preparation also contains thimerosal as a preservative at 0.01w/v%.

[INDICATION] The preparation is used for prophylaxis of pertussis, diphtheria and tetanus.

[ADMINISTRATION] Initial immunization:

Usually three doses of 0.5ml are injected subcutaneously at intervals of 3 to 8 weeks between doses.

Additional immunization:

Usually a single dose of 0.5ml is injected subcutaneously between 12 and 18 months after the initial immunization.

The skim at the site of injection should first be cleansed and disinfected. Shake vial thoroughly before each use. Cleanse top of rubber stopper of the vial with a suitable antiseptic and wipe away all excess. Using a sterile needle and syringe, draw plunger back to the point representing the desired dose then insert needle into vial through center of stopper. Expel air into vial, invert vial and withdraw dose. Eject air bubbles from syringe before drawing needle away from vial.

A separate heat-sterilized syringe and needle or a new disposable equivalent should be used for each individual person to prevent transinission of homologous serum hepatitis and other infectious agents from one person to another.

[CONTRAINDICATIONS] The administration of this vaccine is contraindicated, except when, in the opinion of the physician, withholding the vaccine entails even greater risk.

1. severe febrile illnesses or other active infection
2. illnesses of heart, Kidney or liver
3. diabetes or other malnutritions
4. leukemia, lymphomas and other generalized malignancies
5. hypersensitivenesses
6. pregnancy

[SIDE EFFECTS] The vaccination may be followed by reddening, swelling, pain or indulation at the inoculation site, or by systemic reactions such as fever, but they are all temporary and disappear within 2 - 3 days. Reddening or swelling at the inoculation site may be occasionally seen several days after inoculation. Since the preparation is an adsorbed vaccine containing aluminium, small indulation may persist for one month or so. Vaccinees who receive more than twice may occasionally present remarkable local reactions, which usually disappear within several days.

[CAUTIONS IN DEALING WITH THE VACCINE]

1. The preparation, if frozen by mistake, must not be used, because it may have undergone changes in quality.
2. It is absolutely necessary that the preparation, taken out of the refrigerator and then warmed to room temperature, should be shaken into a uniform solution before use.
3. The vessel, once thrusted with the needle, must be used within that day.

[STORAGE] Avoid exposure to direct sunlight keep 2°C - 10°C without freezing.

[PACKAGE] Vials each containing: 10ml

APPENDIX 9

SOUTH AFRICAN WHOLE-CELL PERTUSSIS VACCINE COMBINED WITH DIPHTHERIA-TETANUS TOXOIDS: PACKAGE INSERT

THE SOUTH AFRICAN INSTITUTE FOR MEDICAL RESEARCH

Hospital Street 2001 | Johannesburg Telegrams: "Bacteria" | P.O. Box 1038 2000

ADSORBED DIPHTHERIA, TETANUS AND PERTUSSIS VACCINE

DTPer/Vac/Ads

This vaccine contains 25 Lf of purified diphtheria toxoid and 6 Lf of purified tetanus toxoid adsorbed on 1,25 mg aluminium phosphate, and 10 000 million *Bordetella pertussis* per 0,5 ml, preserved with 0,01% thiomersal.

It is intended for the immunization of young children against diphtheria, tetanus and whooping cough simultaneously and should not normally be given to children older than 2-3 years.

It should not be injected when the child is ill and should be used with great caution, if at all, if the child is allergic or has suffered from convulsions or if there is a family history of neurological disorder.

Dosage: three intramuscular injections of 0,5 ml with an interval of four weeks between injections.

A further intramuscular reinforcing dose of 0,5 ml may be given 1-2 years later.

GENERAL

Sterilize the rubber closure of the bottle with tincture of iodine. Shake the bottle vigorously before vaccine is withdrawn.

Withdraw only one dose at a time.

Inject intramuscularly and massage the site of injection gently.

Store the vaccine at 2°-10°C. It must not be frozen; this causes irreversible changes in the phosphate gel.

PACKING

Rubber capped bottle containing 20 doses.

Ampoule containing a single dose.

APPENDIX 10

PREPARATION OF FIMBRIAL ANTIGEN, AGG2,3
(A. ROBINSON, L.I. IRONS, BIOLOGICS DIVISION, PHLS, CAMR)

Bordetella pertussis strain Wellcome 28 (stereotype 1,2,3) was grown for 48 hours in a fermenter containing the medium of Stainer and Scholte supplemented with casamino acids and 2-6 dimethyl-beta-cyclodextrin (Imaizumi *et al*, 1984). The cells were recovered by centrifugation and used for the preparation of fimbriae.

AGG 2,3 was prepared by homogenisation of the cells using a Silverson homogeniser and purified from the homogenate by repeated precipitations with 30% and 15% saturated ammonium sulphate.

The preparation was finally obtained as a solution in 5 mM phosphate buffer pH 7.2 containing 50 mM sodium chloride. Lactose was added to 1% (w/v) to the antigen solution. SDS-PAGE analysis and assays for protein and LPS content were performed on the freeze-dried material.

APPENDIX 11

PREPARATION OF REAGENTS FOR ELISA

1. Blocking buffer

pH 7.4

NaCl	8.0 g
KH_2PO_4	0.2 g
$Na_3HPO_4.12H_20$	2.9 g
KCl	0.2 g
BSA	0.5 g
NaN_3	0.2 g

Distilled water q.s. ad 1000 ml

2. Coating buffer (Carbonate-bicarbonate)

pH 9.6 0.05M

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g

Distilled water up to 1 litre

Store at 4°C for 2 weeks

3. Washing buffer

(PBS - Tween 0.01 M PBS pH 7.4 with 0.5 g/l of polysorbate - 20 (eg. TWEEN 20))

NaCl	8.0 g
KH_2PO_4	0.2 g
$Na_2HPO_4.12H_20$	2.9 g
KCl	0.2 g
Tween 20	0.5 ml
NaN_3	0.2 g

Distilled water q.s. ad 1000 ml

Store at 4°C

4. Substrate buffer for anti-tetanus toxoid (ELISA)

Borax anhydrous	3.7 g
Succinic acid	3.7 g
Urea hydrogen peroxide	0.4 g

Distilled water up to 1 litre

The pH of this solution should be approximately 5.0 and may be adjusted to that level using good quality sodium hydroxide or hydrochloric acid if necessary.

5. Tris saline (TS) pH 8.0

Tris buffer (Sigma T-1378)	6.05 g (0.05 M)
Sodium chloride	5.85 g (0.1 M)
Distilled water up to 1 litre	

Adjusted to pH 8.0 using concentrated hydrochloric acid.

6. Substrate buffer for anti-PT, anti-FHA, anti-AGG 2,3 and diphtheria antitoxin ELISA

For 50 ml (sufficient for 5 microtitre plates)

TMB	5.0 mg
DMSO	500 μl

Acetate buffer	5.0 ml
Distilled water	45 ml

Hydrogen peroxide (H_2O_2) 20 μl

Add DMSO to TMB. Shake. Leave in dark. Add water to acetate buffer. To use add DMSO/TMB drop wise to acetate/water solution. Add H_2O_2. Use quickly.

APPENDIX 12

DETERMINATION OF OPTIMAL CONCENTRATIONS OF ANTIGENS FOR COATING OF MICROTITRE PLATES

A "chequerboard" format was used to ascertain the optimum combination of antigen and positive control serum dilutions for performing the assays (Manclark & Meade, 1980).

MATERIALS

1. NUNC Microtitre immunoplates
2. Carbonate-coating buffer (pH 9.6)
3. Antigens
4. Conjugate
5. Positive Reference Serum
6. Washing Buffer (PBS-Tween)
7. Dilution Buffer.
8. Substrate and substrate buffer
9. 5M NaOH
10. ELISA Washer

METHOD

The ELISA is run with the following modifications -

1. *Adsorption:* Six two-fold serial dilutions of coating antigen, from 1:50 to 1:1600 were made in coating buffer. 50 μl of each dilution was used to coat duplicate wells of micro-immunoplates. The initial dilution should result in a protein concentration of 10 μl/ml. The last row contained only coating buffer.

2. Plates were kept overnight at room temperature then washed three times with washing buffer before further use.

3. *Addition of sample:* A volume of 50 µl positive reference serum was added to all wells at a 1:100 dilution in PBS-Tween. Plates were incubated for two hours at room temperature then washed three times.

4. *Addition of conjugate:* A volume of 50 µl/well of optimal dilution of conjugate (as determined above) was added and plates were again incubated for two hours at room temperature. Plates were washed thrice and 100 µl/well substrate solution was added, plates were then incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 10 µl 5M NaOH and absorbance was read after 30 minutes. Graph Optical Density (OD) versus log (antigen concentration). The antigen concentration giving an OD of 1.0 was taken as being the "working antigen concentration".

APPENDIX 13

DETERMINATION OF OPTIMAL CONJUGATE DILUTION BY CHEQUERBOARD TITRATION

The following method was used to determine the dilution of the conjugate needed to generate the expected titres of the reference serum against the test antigens (Manclark & Meade, 1980).

Requirements: All reagents prepared under sterile conditions.

MATERIALS

1. *Coating Buffer:* Bicarbonate buffer - 0.05M pH 0.5
2. *Microtitre plates.*
3. *Antigen:* Antigen was diluted in coating buffer to make a 5 μg/ml solution.
4. *Conjugate:* Dilutions of 1:100, 1:150, 1:200, 1:300, 1:800, 1:1200, 1:1600, 1:2400 and 1:3200 were made up in washing buffer under sterile conditions.
5. *Negative control or blank.*
6. *Positive reference serum:* Diluted to 1:100 in PBS-Tween.
7. *Phosphate Buffer Saline (PBS):* 0.01 M phosphate buffer pH 7.4 containing 0.15 M NaCl and 0.1% NaN_3.
8. *Washing Buffer:* PBS and 0.05% Tween 20 (Polyoxethylene sorbitan monolaurate).
9. *Substrate.*
10. *Substrate Buffer.*
11. *Polystyrene Tubes:* 12 x 75 mm.
12. *5M NaOH.*
13. H_2O: Distilled.

METHOD

Run ELISA with the following modifications -

1. *Adsorption:* The microtitre plates were coated by adding 100 μl diluted antigen solution per well at optimal concentration if known, or 5 μg/ml if unknown. The plates were covered and incubated overnight at room temperature.
2. After washing, 50 μl of positive reference serum was added to all wells, the plate was then incubated at room temperature for three hours then washed again.
3. *Addition of sample:* Positive reference serum was added to all wells at a 1:100 dilution in PBS-Tween.
4. *Addition of conjugate:* Six two-fold serial dilutions of conjugate ranging from 1:50 to 1:1600 were made in PBST and 50 μl of each dilution was added to duplicate wells. Plates were again incubated for three hours and washed as usual.
5. *Addition of substrate:* After washing plates 100 μl/well substrate solution one is added. The reaction was stopped by addition of 10 μl 5M NaOH after ±30 minutes incubation.
6. Absorbance was read after 30 minutes. Graph Optical Density (OD) vs. log conjugate dilution. Conjugate dilution giving OD of 1.0 is the working dilution of conjugate.

APPENDIX 14

PREPARATION OF REAGENTS FOR SDS-PAGE AND IMMUNOBLOTTING

STACKING GEL

0.25M TRIS Buffer with 0.2% SDS	3 ml
Solution of 40% sucrose, 8% acrylamide and 2% bis	3 ml

Deaerate, then add:

TEMED (BDH)	6 µl
10% Ammonium persulphate	60 µl (freshly prepared)

RESOLVING GEL

Buffer 0.75M TRIS, 0.2% SDS	20 ml
Distilled water	7 ml
30% acrylamide, 0.8% BIS	13 ml
TEMED	30 µl
10% Ammonium persulphate	300 µl

BUFFER FOR BLOTTING CHAMBER

Glycine	72 g
TRIS	15 g
Methanol	1 litre

Make up to a final volume of 5 litre with distilled H_20. Store at 4°C.

SUBSTRATE

4-chloronaphthol	10-20 mg
Methanol	1 ml
Add 400 µl of above drop wise to	
PBS	40 ml
Add H_20_2	10 µl

ELECTROPHORESIS BUFFER (pH 8.3-8.4)

Glycine	72 g
TRIS	15.15 g
SDS	5 g

Make up to 5 litre with distilled H_20.

COOMASSIE BLUE (CB)

0.05% CB		0.025% CB
0.25g	CB	0.0125 g
50 ml	glacial acetic acid	50 ml
125 ml	isopropyl alcohol	40 ml
400 ml	distilled H_20	400 ml

Filter.

TRIS/SDS/MERCAPTOETHANOL/GLYCEROL(TSMG)

TRIS	18.93 g
Distilled H_20	to a final volume of 150 ml

Bring pH to 6.8 with c.HCl

Glycerol	250 ml (omit if antigen solution contains glycerol)
SDS	50 g
Bromophenol blue	0.025 g

Bring final volume to 450 ml; then add 90 ml of this solution to 10 ml mercaptoethanol.

APPENDIX 15

SOUTH AFRICAN DIPHTHERIA-TETANUS TOXOIDS VACCINE: PACKAGE INSERT

THE SOUTH AFRICAN INSTITUTE FOR MEDICAL RESEARCH

Hospital Street 2001 | Johannesburg Telegrams: "Bacteria" | P.O. Box 1038 2000

TETANUS VACCINE

Tet/Vac/FT

This vaccine contains 10 Lf of purified formol-toxoid (FT) and 0,01% thiomersal in 0,5 ml.

It has largely been superseded by 'Adsorbed Tetanus Vaccine' in which the toxoid is adsorbed on aluminium phosphate. The adsorbed vaccine is the antigen of choice for primary immunization and **must** be used when immunization is begun in conjunction with passive protection by means of Tetanus Antitoxin.

Either of the two vaccines may be used for the purpose of boosting already established immunity.

Unimmunized persons should receive three subcutaneous injections of Tetanus Vaccine (FT), each of 0,5 ml. The recommended interval between the first and second dose is from six to eight weeks and between the second and third, six months.

A booster dose of 0,5 ml should be given every 10 years and immediately after an injury if a year or more has lapsed since the last injection.

Sterilize the rubber closure with tincture of iodine.

Store at 2°-10°C.

ADSORBED DIPHTHERIA VACCINE

Dip/Vac/Ads

This vaccine contains 0,5 ml 25 Lf of purified diphtheria toxoid adsorbed on 2,5 mg aluminium phosphate. It is preserved with 0,01% thiomersal.

Basic immunization may be achieved with two 0,5 ml doses given intramuscularly with an interval of six weeks. Those immunized during their first year of life should receive a booster dose of 0,5 ml one to two years later.

The following vaccines may sometimes be used with advantage. Adsorbed Diphtheria and Tetanus Vaccine (DT/Vac/Ads) and, for young children only, Adsorbed Diphtheria, Tetanus and Pertussis Vaccine (DTPer/Vac/Ads).

Infants are unlikely to show sensitivity reactions, but older children, adolescents and adults should preferably be Schick-tested to determine immunity and sensitivity before immunization with any vaccine containing diphtheria toxoid is undertaken.

GENERAL

Sterilize the rubber closure of the bottle with tincture of iodine.

Shake the bottle vigorously before vaccine is withdrawn.

Withdraw one dose only at a time.

Inject intramuscularly and massage the site of injection gently.

Store the vaccine at 2°-10°C. It must not be frozen; this causes irreversible changes in the phosphate gel.

PACKING

Rubber capped bottle containing 20 doses.

Ampoule containing a single dose.