

A BIOCHEMICAL AND IMMUNOLOGICAL COMPARISON OF
THE JAAGSIEKTE AND TWO
RELATED RETROVIRUSES

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ABSTRACT

Jaagsiekte is a contagious cancer affecting the lungs of sheep. Although the etiological agent is Jaagsiekte retrovirus (JSRV), two other retroviruses viz South African maedi visna virus (SA - OMV) and a novel Bovine retrovirus (BRV) have been associated with or implicated in the jaagsiekte disease complex.

JSRV was sufficiently purified from lung rinse material using a Freon extraction, Percoll density gradient centrifugation and chromatography on a Sephacryl column, its polypeptide composition was studied by gel electrophoresis and its morphology observed electron microscopically. Monoclonal antibodies were made against purified preparations of the virus. Two hybridomas were isolated that produced MAbs which appear to be tumour cell specific. A third hybridoma, called 4A10, produces antibodies considered to be viral specific. These MAbs have been used in the development of JS specific immunoassays. A cross reaction between JSRV and a polyclonal serum against Mason Pfizer monkey virus (MPMV) was confirmed and used in a Western blot technique to identify, monitor and differentiate JSRV from other viruses.

During the study of JSRV it became apparent that another retrovirus was often present in JS infected lungs. This virus, referred to as SA - OMV I, is a novel South African isolate of maedi - visna virus (MVV). As SA - OMV I has physicochemical characteristics similar to JSRV, it was often found in purified JSRV preparations. Being a retrovirus it is also detected by the reverse

transcriptase assay which was the only method used to assay and monitor for JSRV during the early stages of our work. Using a Western blot technique and sera against MVV and MPMV it was possible to simultaneously detect and differentiate JSRV from SA - OMV I. A method was also developed whereby the two viruses could be separated from each other during purification.

The information gained and techniques developed whilst studying JSRV were also used to isolate and characterize BRV. This novel virus originated from bovine cells that had been co-cultivated with white blood cells from an ox suffering from malignant catarrhal fever. Three out of four sheep inoculated with BRV developed JS. It therefore had to be ascertained whether this virus was related to JSRV or not. The comparative study revealed that BRV was biochemically and morphologically quite different from JSRV. Interestingly, it was shown that serum against MPMV cross reacted with a 32 kd protein of BRV indicating a serological relationship between JSRV, MPMV and BRV. The possible role of BRV in the etiology of jaagsiekte remains to be elucidated.

PREFACE

The experimental work described in this was carried out in the department of Molecular Biology, Veterinary Research Institute, Onderstepoort, from February 1984 to December 1987 under the supervision of Dr. D.W. Verwoerd (Veterinary Research Institute, Onderstepoort) and Dr. C. Dennison (University of Natal, Pietermaritzburg).

These studies represent original work by the author and have not been submitted in any other form to another University. Work of other authors has been duly acknowledged in the text.

(Denis Francis York)
15th day of December 1987

THIS THESIS IS DEDICATED

TO MY WIFE

MIKAELA

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LIST OF PUBLICATIONS

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- QUERAT, G., BARBAN, V., VIGNE, R., PAYNE, A-L., YORK, D.F., DEVILLIERS, E-M. & VERWOERD, D.W. (1987). Virology. Characteristics of a novel lentivirus derived from South African sheep with pulmonary adenocarcinoma (jaagsiekte) 158, 158-167.
- TUSTIN, R.C., PAYNE, A-L., YORK, D.F. & VERWOERD, D.W. (1988). Experimental transmission of jaagsiekte (ovine pulmonary adenomatosis) to goats. Onderstepoort Journal of Veterinary Research. 55, (in press).
- VERWOERD, D.W., PAYNE, A-L., YORK, D.F. & MYER, M.S. (1983). Isolation and preliminary characterization of the jaagsiekte retrovirus (JSRV). Onderstepoort Journal of Veterinary Research. 50, 309-316.

ABBREVIATIONS

The following abbreviations are used in the text:

Viruses:

BLV - bovine leukemia virus; BSV - bovine syncytial virus; HIV - human immunodeficiency virus; HTLV - human T-cell leukemia virus; LV - lentivirus; MCFV - malignant catarrhal fever virus; MMTV - mouse mammary tumour virus; MPMV - Mason Pfizer monkey virus; MuLV - murine leukemia virus; MVV - maedi-visna virus; SA-OMVV I & II - South African maedi-visna virus type I & II; SMRV - squirrel monkey retrovirus.

Terms:

Abs - absorbance; Ag - antigen; Ab - antibody; ELISA - enzyme linked immunosorbent assay; env - envelope; gag - group antigen; gp - glycoprotein; h - hour; JS - jaagsiekte; kb - kilo base; kd - kilo dalton; M - molar; MAbs - monoclonal antibodies; min - minutes; NL - normal lung; OD - optical density; p - polypeptide; PAGE - polyacrylamide gel electrophoresis; pfu - plaque forming units; poly-A - poly adenylated; Rm - relative mobility; pol - polymerase; RDP - RNA dependant DNA polymerase (reverse transcriptase); TEM - transmission electron microscopy; VRI - Veterinary Research Institute;

Buffers:

DMEM - Dulbecco's modified Eagle's medium; PBS - phosphate buffered saline (see Appendix I); PSB - protein suspension buffer (see 2.8.1.1);

Chemicals:

BSA - bovine serum albumin; DTT - dithiothreitol (Boeringer Mannheim Biochemica); EDTA - ethylenediaminetetra-acetic acid disodium salt (Sarchem Pty Ltd); FCA - Freund's complete adjuvant (Difco Laboratories); FCS - foetal calf serum (Highveld Biologicals Pty Ltd); FICA - Freund's incomplete adjuvant (Difco Laboratories); HAT - hypoxanthine aminopterin thymidine (Flow laboratories Pty Ltd); HS - horse serum (VRI); HT - hypoxanthine thymidine (Flow Laboratories); PEG - polyethyleneglycol (Merk, Schuchardt and Co.); PTA - phosphotungstic acid; SDS - sodium dodecyl sulphate (Merk, Schuchardt and Co.). UA - uranyl acetate.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 DEFINITION AND DESCRIPTION OF JAAGSIEKTE

Jaagsiekte (JS), or sheep pulmonary adenomatosis, is a specific contagious disease of sheep which is characterized by the progressive development of a primary lung neoplasm (Tustin, 1969). The name jaagsiekte is derived from the descriptive Afrikaans term meaning "accelerated breathing".

The major clinical signs are spasmodic bouts of coughing and marked respiratory distress which is especially evident after strenuous activity, when respiratory movements become short and jerky and dyspnoea becomes progressive. Moist rales are heard on auscultation of the chest and sometimes without the aid of a stethoscope. There is a great increase in the amount of secretion from the lungs and if the animal is up-ended, with the head down, fluid streams out of the nostrils. This is regarded as a pathognomonic symptom of JS and has been reported by a number of early investigators, including Mitchell (1915) and Dungal, Gislason and Taylor (1938). However, the amount of fluid recovered in this manner varies and can be very little in some cases (Stevenson, 1969).

JS itself is not pyrogenic, but fever may occur as a result of secondary bacterial infections. If the sheep are well cared for, they may survive for up to a year after the first clinical signs are observed. However, experimentally infected new-born lambs

often die within a few days after showing clinical symptoms (Verwoerd, Tustin and Payne, 1985).

1.2 DISTRIBUTION

The earliest record of JS appeared in a letter written in 1825 by Veldkornet P. Aukamp in the Rhenoster district of the Cape of Good Hope. He reported that 800 of his sheep had died of a strange disease called "jaagziekte" which appeared to be contagious with a 100% mortality rate (from Tustin, 1969). Since then there have been numerous reports from all over the world. JS was first described in England by McFaydean (1894), in Germany by Eben (1899), in France by Aynaud (1926) and in various countries since then. Today the world distribution includes Kenya, Peru, Chile, India, Israel, Turkey, Iceland, The United Kingdom, Bulgaria, Yugoslavia, S.E. Russia, Greece, Italy, Spain (Tustin, 1969), Portugal, Czechoslovakia, Rumania, European Russia, Tanzania, United States (Wandera, 1971), Canada (Stephenson and Rehmtula, 1980), Iraq (Al-Zubaidy and Sokkar, 1979), Switzerland (Tontis, Besetti, Konig and Luginbahl, 1979), the Netherlands (Houwers and Terpstra, 1984) and North America (DeMartini, Rosadio, Sharp, Russel and Laimore, 1987). It is unexplained why it has not been reported in Australia and New Zealand. Both countries have imported sheep via Britain and South Africa from Spain (Wandera, 1971) which is thought to be the possible origin of JS in South Africa (Tustin, 1969).

1.3 INCIDENCE AND ECONOMIC IMPORTANCE

The incidence of JS in a flock varies depending upon the susceptibility of the population, flock management and possibly the breed of sheep (Verwoerd et al., 1985). It is initially very high in flocks that have not previously been exposed to JS. A classic example of this is the experience suffered by the farmers in Iceland during the 1930's and 1940's when large scale outbreaks of JS occurred (Dungal et al., 1938 and Dungal, 1946).

A Karakul ram was imported into Iceland from Germany during December, 1933. After a few weeks quarantine it was put out to serve ewes. A year and a half later, between April and December 1935, the farmer lost 259 out of 475 (54%) of his ewes. The disease spread to other flocks and by 1938 flocks in nearly all of Iceland were affected, the incidence being as high as 50 - 60% in the course of two years. The disease was at its height from 1936 to 1945, but tended to become more rare in subsequent years (Sigurdsson, 1954b). In Iceland, the Gottorp breed of sheep was the most susceptible. Unfortunately, it was also the most popular and some farms lost as many as 90% of this breed. The Adalbol breed, on the other hand, proved to be conspicuously resistant. On some severely affected farms only 10% of this breed were infected.

The actual number of JS infected animals in JS flocks has varied in different countries. Where outbreaks were spasmodic and where most cases were only seen in abattoirs, the percentage was generally low, such as 0,1% in Chile; 0,2% in Northern Germany; 0,08%

in Spain; 0,6% in Peru (Wandera, 1971) and as low as 0,033% in China (Deng, Zhang, Han and Bal, 1981). In Britain the incidence was less than 1%, but the disease now appears to be on the increase as the number of JS diagnosed cases doubled between 1975 and 1982, from 2,1% to 4,1% incidence (Ross and Williams, 1983).

The disease is also of economic importance in Bulgaria. When first reported in 1955 (Enchev, Tomov and Ivanov, 1958), it was stated that affected flocks were experiencing losses of between 1 and 3%. Following spread to other regions of that country an epidemic occurred with losses reaching 35,5% on certain farms (Enchev, 1961).

In the 1950's Kenya had up to 30% annual mortality from JS alone on certain farms (Shirlaw, 1959). It has since dropped to between 1 and 5% in flocks where it is endemic (Wandera, 1971).

In a recent survey in Scotland, 20% of all mature sheep necropsied at the Veterinary Investigation Centres had JS (Hunter and Munro, 1983). In South Africa there are some indications of a lower incidence in the English breeds than in their Merino and Karakul counterparts and their cross breeds which make up the bulk of the sheep population (Verwoerd et al., 1985). The incidence of JS in South Africa varies between farms, from less than 1% to about 20%. However, losses of 30% and more in certain flocks were not unusual when the disease was first introduced into South Africa in the early 1800's (Tustin, 1969).

1.4 JAAGSIEKTE IN GOATS

Although JS is essentially a sheep disease, there have been reports that it occurred naturally in goats (Nobel, 1958; Cuba-Caparo, De La Vega and Copaira, 1961). These reports were not convincing because of the inadequate differentiation between alveolar epithelialization and true adenocarcinomatous lesions (Tustin, 1969). However, recent reports have confirmed that there is a low incidence of JS in goats (Banerjee and Gupta, 1979; Sriraman, Pao and Naidu, 1982). The disease was also recently experimentally transmitted to a goat kid (Sharp, Angus, Jassim and Scott, 1986) and confirmed in this laboratory where two out of five goat kids developed JS lesions after inoculation with JS lung wash pellet (Tustin, Williamson, York and Verwoerd, 1988). In comparison, the goats were very much less susceptible to JS than sheep (Tustin et al., 1988).

1.5 TRANSMISSION OF JAAGSIEKTE

The first attempts to transmit JS were made by Hutcheon in 1903 and Robertson in 1904. Both reported unsuccessful attempts to transmit the disease by cohabitation of infected and healthy sheep and by inoculation of healthy sheep with JS affected blood and tissues. In 1915, Mitchell reported that he had transmitted the disease. However, in 1929 De Kock disclaimed this fact, arguing that it was possibly "Graaff-Reinet" disease which Mitchell had transmitted, as the lesions described by Mitchell in 1915 were similar to those of this disease. "Graaff-Reinet" disease is a

form of pneumonia and is pathologically distinct from JS (Tustin, personal communication).

De Kock (1929) also attempted to determine whether JS was infectious by exposing healthy sheep to infected sheep. Although he did transmit the disease to a small number of animals, he was unaware that it was JS at that stage, because the lesions were small, localized and asymptomatic. Dungal (1946) confirmed that JS could be transmitted by cohabitation of healthy with diseased sheep. He also demonstrated that the exhaled respiratory air and bronchial secretions of affected sheep contained the etiological agent. By maintaining healthy sheep 1,5 yards above the heads of diseased sheep, which were kept in a lower compartment, he excluded faeces and urine as possible sources of infection and showed that three out of eight lambs in the above chamber contracted JS. In another experiment he made a diseased sheep breathe through a glycerine/saline solution, which when injected into three lambs, caused JS in two of the lambs. In a similar experiment, but after filtering the glycerine/saline solution through a gradocol membrane with pores 9 μ m in diameter, he produced the disease in one out of four lambs. The disease was also successfully transmitted by exposing sheep to aerosol sprays of infectious material (Dungal, 1946; Markson and Terlecki, 1964).

1.6 ETIOLOGY OF JAAGSIEKTE

Even though the above findings suggested a viral etiology, other

agents such as Mycoplasma spp (Mackay, Nisbet and Foogie, 1963) and Chlamydia (Wandera, 1971) were also considered as possible agents.

For a long time Herpesvirus ovis was regarded as the causal agent. By 1975 ovine herpes virus had been isolated from JS tumours throughout the world, viz. Scotland (Smith and Mackay, 1969), Yugoslavia (Cvjetanovic, Forseck, Nevjestic and Rukanvia, 1972), Kenya (Malmquist, Krauss, Moulton and Wandera, 1972) and South Africa (De Villiers, Els and Verwoerd, 1975). However, all attempts to transmit the disease with herpes virus isolates failed. Yet, it was demonstrated that the herpes virus and a retrovirus of JS infected sheep caused more and greater areas of adenomatous change, than the retrovirus alone (Martin, Scott, Sharp, Angus and Norval, 1976). A few years later, Martin, Angus, Robinson and Scott (1979) suggested that the herpes virus acted synergistically in some way to cause or enhance the oncogenic potential of the retrovirus. They went further and claimed that the retrovirus could play the principal role in the production of JS, but may be aided by the herpes virus. However, molecular hybridization studies failed to reveal any correlation between the herpes virus DNA sequences in the cell genome and the occurrence of the disease (De Villiers and Verwoerd, 1980). From their observations, they excluded any role for the herpes virus in the etiology of JS, except possibly as a helper function or playing some role during transformation.

The first indication of a possible retrovirus involvement in the

etiology of JS was the observation of a particle possessing type C viral morphology in JS infected lungs (Perk and Hod, 1971). A year later Malmquist, Krauss, Moulton and Wandera (1972) reported a virus in cell cultures, established from JS infected lungs, with morphological characteristics of a typical retrovirus. Two years later Perk, Michalides, Spiegelman and Scholm (1974) published biochemical evidence for the presence of particles with reverse transcriptase activity in lung extracts. They used a technique that simultaneously detected two features diagnostic of the known retroviruses viz. a 60 - 70S RNA and an RNA dependant DNA polymerase (reverse transcriptase). They also found retrovirus-like particles in sheep lungs with JS but not in tissue from normal lungs.

However, neither Perk et al., (1974) nor Malmquist et al., (1972) carried out any transmission studies. In 1976, Coetzee, Els and Verwoerd transmitted JS to three out of eight lambs using an epithelial cell line that had been established in culture from JS lesions. Although it was not realized at the time, these cells were later shown to contain jaagsiekte retrovirus-like particles.

In 1976, Martin and colleagues demonstrated some correlation between reverse transcriptase in the inoculum and the transmission of JS. Four years later Verwoerd, Williamson and De Villiers (1980) isolated a virus with biochemical characteristics of a retrovirus. The following year Verwoerd and Williamson (1981), demonstrated an inverse dose relationship between the amount of

reverse transcriptase activity inoculated into lambs and the time taken to produce clinical symptoms of JS. These transmission studies were supported with electron microscopy studies which revealed that the virus used to inoculate the lambs was identical to the virus seen in the resulting diseased lung tissue. Virus isolated from natural cases was also shown to be identical to the jaagsiekte virus used experimentally (Payne, Verwoerd and Garnett, 1983).

The infectious lung fluid from JS infected sheep contained particles which had biochemical characteristics of retroviruses, viz. a bouyant density of 1,186 g/ml in sucrose and reverse transcriptase activity at this density (Verwoerd, Payne, York and Myer, 1983). Particles at this density have also been shown to cross-react with serum against the group-specific antigens of both mouse mammary tumour virus (MMTV) and Mason-Pfizer monkey virus (MPMV), which are the prototypes of type B and D oncoviruses, respectively (Sharp and Herring, 1983). However, these particles did not cross-react with sera against the group-specific antigen of maedi-visna virus, the prototype of the lentivirus subfamily (Payne, York, De Villiers, Verwoerd, Querat, Barban, Sauze and Vigne, 1986).

1.7 LENTIVIRUS AND JAAGSIEKTE

At this stage the evidence was very convincing that JS was caused by a retrovirus. However, besides the purported JS virus, another retrovirus is found in sheep. This second retrovirus, called maedi-visna virus, causes a progressive interstitial pneumonia in

sheep lungs. The virus, often referred to as the maedi-visna virus (MVV) complex, forms the prototype of the subfamily Lentivirinae (Matthews, 1979). Maedi meaning "shortness of breath" and visna meaning "wasting", were initially recognized in Iceland as two distinct clinical syndromes involving the ovine lung and brain, respectively (Sigurdsson, 1954 a, b and c). It is now accepted from comparative studies using morphogenesis, molecular hybridization techniques, serology and animal experiments that both diseases are caused by the same virus (Harter and Coward, 1974; Weiss, Sweet, Gulati and Harter, 1976 and Stowering, Haase and Charman, 1979).

JS has been closely associated with maedi in various parts of the world. The two diseases were introduced into Iceland at the same time and possibly by the same imported animal (Palsson, 1976). In Peru, both diseases were demonstrated in the same sheep (Snyder, DeMartini, Ameghino and Caletti, 1983), whilst both were shown to be present in the same flock in Kenya (Wandera, 1971), the Netherlands (Houwers and Terpstra, 1984) and in England (Markson, Spence and Dawson, 1983). It is reported in this thesis that both viruses are also present in the same sheep in South Africa. Maedi, and the associated neurological affection, visna, in their classical forms, had never been diagnosed in South Africa (Verwoerd, personal communication). However, in early descriptions of the histopathological lesions of JS (Mitchell, 1915), it was clear that interstitial pneumonia was also present in some of the JS lesions. After comparing his own observations with early descriptions of JS, De Kock, as early as 1929, concluded that there may be two

specific diseases in sheep. A "papilliform cystadenoma" (JS) and a chronic indurative "catarrhal pneumonia", which he called "Graaff-Reinet" disease. Maedi-visna has also been described under various other names: Montana sheep disease, or Ovine progressive pneumonia in the United States, Zwoegersiekte in Holland and La Bouhite in France. However, clinical, histopathological and virological studies indicate that these pulmonary conditions are all caused by maedi-visna virus (Perk, 1982). Since the early report by De Kock of Graaff-Reinet disease, it has not been described again in South Africa.

Up until 1983, South Africa was therefore considered to be free of maedi-visna virus. This assumption was based on clinical and pathological observations (Verwoerd et al., 1983) and the negative results of a serological survey of a few sheep farms. However, the isolation in our laboratory of a MVV-like virus from a culture that had been infected with JS lung wash material, prompted an investigation into the presence of this virus in our experimental sheep.

After the development of an immunoblot technique to identify MVV antibodies (York, 1984), the sera of our experimental sheep were tested. The results revealed that 85,2% (23 out of 27) of the sheep had MVV antibodies. MVV-like virus was also isolated from macrophage cultures from 14 out of 16 sheep that had JS (Payne et al., 1986). Recently, DeMartini, Rosadio, Sharp, Russell and Lairmore, (1987) also reported the isolation of MVV from 6 out of 7

lambs experimentally infected with JS.

Although a lentivirus has been found together with JS in the same lung, infectivity studies have shown that it does not cause JS. In this laboratory eighteen lambs were injected with cell cultured lentivirus and kept isolated from all other sheep. After two years none of the sheep had developed JS (Payne et al., 1986). Lentiviruses, of which MVV is the prototype, are in any case non-transforming (Matthews, 1982). Speculation on the possible role of the lentivirus in JS will be considered in the general discussion of this thesis.

1.8 BOVINE RETROVIRUS (BRV) AND JAAGSIEKTE

During the course of this study on the JS disease complex, yet another virus was implicated in the etiology of JS. This virus was isolated from a calf foetal thyroid (CFTH) culture that had been co-cultured with white blood cells from an ox suffering from the sheep associated form of Malignant Catarrhal Fever (Barnard, personal communication). This disease is thought to be caused by a herpes virus (Plowright, 1984). However, the morphology of the cell cultured virus, as revealed by transmission electron microscopy, more closely resembled that of the retrovirus family.

To eliminate the possibility that the isolated virus was bovine leukemia virus (BLV), the most common bovine retrovirus, sheep were injected with the virus and tested for seroconversion, a standard method to identify BLV. However, none of the sheep produced antibodies to BLV. Interestingly though, one of the sheep

developed JS. At this stage Dr. Barnard, who isolated this virus at Onderstepoort, handed it over to our department for further investigation.

In chapter 5 the biochemical, immunological and morphological characteristics of this virus and its comparison with JSRV are presented. In addition, the results of further infectivity studies are also mentioned. This virus has provisionally been named BRV (bovine retrovirus). It is interesting that whilst this thesis was being written, Amborski, Storz, Keney, Lo and McChesney (1987) published a paper on the isolation of a retrovirus from an American bison. The bison also showed clinical symptoms of Malignant Catarrhal Fever. Their studies were, however, not very detailed so it is difficult to ascertain whether there may be any similarities between the two viruses. They have not implicated their virus in the etiology of any disease.

1.9 THE FAMILY RETROVIRIDAE

The common factor between JSRV, MVV and BRV is that all three are retroviruses and that they have all, in some way, been implicated in the JS disease complex. A discussion of the retroviruses, in general, is therefore relevant.

Retroviruses are very widely distributed in nature (Teich, 1982). Members of this family are recognized by their morphology, the structure of their RNA viral genomes and the presence of an RNA dependant DNA polymerase (reverse transcriptase) (Lowry, 1985).

Retroviruses are divided into three subfamilies of unequal size: Oncovirinae from Greek "onkos" meaning tumour, Lentivirinae from Latin "lenti" meaning slow and Spumavirinae from Latin "spuma" meaning foamy. "Retro" refers to the reverse transcriptase and in Latin means backwards (Matthews 1982).

The Spumavirinae have been isolated principally as contaminants of primary tissue culture cells. These viruses derive their name from the characteristic "foamy" degeneration they induce in cultured cells. Spumaviruses have been found in a number of mammalian species, including feline, bovine, non-human primates and humans. They may produce persistent infection, but they have not yet been associated with a distinct pathological entity (Hooks and Gibbs, 1975). The members isolated from monkeys and humans are referred to as foamy viruses, whereas those from cattle, cats and hamsters are referred to as syncytial viruses (Rowson, Rees and Mahy, 1981).

The spumaviruses contain a 60 - 70S RNA genome, an RNA dependant DNA polymerase (reverse transcriptase) and morphologically resemble the RNA tumour viruses (Matthews, 1982). Distinctive features include: electron-lucent nucleoids, long spikes projecting from the surface and maturation by budding into intracytoplasmic vacuoles (Hooks and Gibbs, 1975).

Spumaviruses replicate only in dividing cells. Early events, such as adsorption to and penetration of the cell membrane, are similar

to other enveloped viruses. After uncoating, genetic information is transferred to a DNA intermediate. Infectious DNA can be isolated from infected cells, but little is known of its size or structure. Viral DNA serves as template for synthesis of RNA and virus polypeptides. The polypeptides are first found in the nucleus, then in the cytoplasm and finally in the plasma membrane. A single cycle of replication is completed in 5 - 7 days, with a latency period of 24 hours (Matthews, 1982). Spumaviruses do not induce transformation of cells in vitro, or cause tumours in animals. Bovine syncytial virus (BSV) is a member of this group.

The lentivirus subfamily has been mentioned earlier. The prototype of this group is maedi-visna virus. This group is very similar to the type C oncoviruses in morphology, physical properties and chemical composition. A recent addition to this group is the human immunodeficiency virus (HIV), the causative agent of AIDS (Popovic, Sargadharan, Read and Gallo, 1984). Members of this group have surface projections which are composed of a glycoprotein (gp135 in the case of MVV) that induces type-specific neutralizing antibodies. There are three major internal structural polypeptides, viz. p30, p16 and p14; p30 bears group-specific antigenic determinants that are shared by other members of the subfamily, but not with oncoviruses or spumaviruses (Vigne, Filippi, Querat, Sauze, Vitu, Russo and Delori, 1982). The reverse transcriptase is composed of two subunits with an estimated molecular weight of 70 000 dalton (Lin and Papini, 1979). The nucleoid contains reverse transcriptase, a linear sense single stranded RNA which is not infectious and is polyadenylated at the 3' end

(Haase, 1975). Lentiviruses are horizontally transmitted in infections limited to sheep. No evidence exists for endogenous lentiviruses or endogenous viral genes (Matthews, 1982).

The subfamily Oncovirinae is by far the largest subfamily of retroviruses and has been isolated from virtually all vertebrate species, including humans. Members of this family are divided into four groups, viz. type A, type B, type C and type D retroviruses (Lowry, 1985). They differ with regards to morphology, morphogenesis and polypeptide composition (Dalton, Heine and Melnick, 1975). Within these broad groups, they are further classified by their host range, interference patterns, genetic content, antigenic relatedness, mode of transmission and pathogenicity (Lowry, 1985). An additional distinguishing feature is the cation preference of the reverse transcriptase enzyme of these retroviruses. Mammalian type C viruses have a preference for manganese, whereas types B and D retroviruses, as well as BLV, have a preference for magnesium (Dahlberg, Tronick and Aaronson, 1980; Ferrer, 1980).

The original classification of oncoviruses was based on variations in the electron microscopic appearance and cellular location of the viral particles (Bernhard, 1960).

Type A particles are only found intracellularly and are not as infectious as the mature extracellular forms (Lu, Soong and Wong, 1979). These particles lack the lipid containing outer envelope, as they do not bud from the membrane. There are two types of A particles: intracisternal and intracytoplasmic. The intracisternal

forms are derived from endogenous viral genomes and are apparently replication defective (Kuff, Smith and Lueders, 1981). Most intracytoplasmic type A particles are precursor forms of B and D type particles.

Type B particles, the prototype of which is mouse mammary tumour virus (MMTV), develop their inner core in the cytoplasm and acquire their envelope as they bud from the plasma membrane. The mature particles, which measure about 125 nm in diameter, contain an eccentrically located electron dense core and have long spikes on their surface (Karmarsky, Sarkar and Moore, 1971).

In MMTV, the polypeptide pattern consists of six major proteins. Two are glycosylated and have molecular weights of 52 000 and 36 000 thousand dalton. The other four proteins have molecular weights of 38 000, 23 000, 14 000 and 10 000 dalton (Stephenson, DeVare and Reynolds, 1978).

Type C viruses such as Rous sarcoma virus (RSV), murine leukemia virus (MuLV) and human T-cell leukemia virus (HTLV), develop only as they bud from the plasma membrane. The mature extracellular C type particles are 80 - 100 nm in diameter, with a centrally placed core inside the envelope (Lowry, 1985).

If MuLV is used as the representative of type C oncoviruses, then the polypeptide composition is as follows. There are four gag proteins, viz. p30, p15, p12 and p10. These originated from a

precursor polypeptide of molecular weight 65 000 dalton. The envelope protein originates as a 90 000 dalton precursor, which is cleaved to a gp70 and p15E (Dickson, Eisenman, Fan, Hunter and Teich, 1982).

The type D viruses, of which MPMV is the prototype, develop morphologically in the same way as the type B particles, in that their inner core is assembled in the cytoplasm. However, the mature (budded) D type particles resemble C type particles more closely (Chapman and Mason, 1970). They are also genetically more closely related to the C type viruses (DeVare, Hanson and Stephenson, 1978).

MPMV contains five non-glycosylated structural proteins with molecular weights of 27 000, 16 000, 14 000, 12 000 and 10 000 dalton (Bradac and Hunter, 1984). In addition, MPMV has two glycosylated envelope proteins of 70 000 and 20 000 dalton (Schochetman, Kortright and Schlom, 1975). MPMV also has a virion-associated reverse transcriptase with a molecular weight of 80 000 dalton (Colcher and Schlom, 1977).

Retrovirus encoded proteins are named according to their size, as estimated from their migration rate in SDS-gels (Dickson and Peters, 1982), modifications such as phosphorylation or glycosylation, and gene of origin. As an example, a 29 000 dalton gag encoded protein is called p29^{gag} and a 70 000 dalton env encoded glycoprotein is called gp70^{env}. However, this only becomes possible when the genome and proteins have been well studied. In

the case of JSRV, BRV and the viruses mentioned in this thesis, the simplified coding method described by August, Bolognesi, Fleissner, Gilden and Nowinski (1974) is used. The coding is very straightforward and is also based on the rate of mobility of the proteins in SDS-gels. Briefly, the prefix represents polypeptide and the suffix represents molecular weight $\times 10^{-3}$, for example p29. In the case of glycosylated proteins the prefix 'gp' is used.

Retroviruses contain only four identifiable genes (protein coding domains). These fall into two classes: those that are essential for replication and the v-onc genes, which enhance the oncogenicity of the virus but are not essential for virus replication (Lowry, 1985). The basic genetic information for the production of infectious progeny virus consists of 3 genes: gag, coding for internal non-glycosylated proteins of the virion, pol, coding for the reverse transcriptase and env, coding for the envelope glycoproteins of the virion. Their left to right (5' to 3') order is gag - pol - env (Matthews, 1982).

These three genes are bounded at each end by genetic sequences termed U5 (at the 5' end of the genome) and U3 (at the 3' end). A small directly repeated element (R) is found at the end of each of these sequences. During the retrovirus replication cycle, the U5, U3 and R element form the large, directly repeated structure found at either end of the retroviral genome, the long terminal repeats (LTR's). They are important for viral replication and essential for stable integration into the host cell genome (Evans and

Lennox, 1985).

Most retroviruses have intact gag, pol and env genes and do not contain a v-onc gene. These viruses are classified as replication competent but transformation defective (v-onc⁻). Those viruses that lack one or more of the three replication genes are replication defective. However, these defective genomes can be rescued by replication competent viruses so that they can infect cells and undergo a replicative cycle (viral DNA synthesis, integration of the viral DNA and expression of their viral genome) that is normal, except for their inability to synthesize all gene products required for making progeny virus. In the absence of a competent virus, these defective viruses will remain as proviruses in the cells until reinfection occurs with a replication competent (helper) virus (Lowry, 1985).

On reinfection of the cells with a replication competent or helper virus, these defective viruses will become infectious. If the replication defective virus has a v-onc gene which codes for a protein that has the ability to transform the cells, the expression of this protein ensures the replication of the cell and therefore expression of the defective genome (Lowry, 1985).

It is now realized that most of the transformation competent ⁺(v-onc) retroviruses are replication defective (Evans and Lennox, 1985). Although it is possible for a v-onc gene to be the fourth gene of a replication competent virus, this usually does not occur. However, there are some isolates of Rous sarcoma virus that

are replication and transformation competent (Bishop and Varmus, 1982).

The transforming (v-onc⁺) retroviruses are quite common and have been found in tumour tissue of many species, including birds, rodents, felines and non-human primates. Under natural conditions these v-onc⁺ viruses are usually not transmitted to a different species (Bishop, 1983).

To date, 21 v-onc genes have been isolated. These genes are derived from highly conserved eukaryotic cellular c-onc genes (Land, Parada and Weinberg, 1983). These normal cellular counterparts of v-onc genes (proto-oncogenes) were initially identified by finding that the normal cells of many species contained sequences and proteins homologous to v-onc genes and to their protein products. A number of different ways by which the products of viral oncogenes might act have been proposed, e.g. phosphorylation of either proteins or phospholipids (Hunter and Cooper, 1985), initiation of DNA synthesis (Martin, 1981) or regulation of transcription (Kingston, Baldwin and Sharp, 1985).

An oncogene must be involved in the JS disease complex. Whether it is part of the JSRV genome or in the type II cells (the transformed cell in jaagsiekte) is uncertain at this stage. Attempts have been made to determine whether JSRV has a gene sequence similar to that of the more common oncogenes, so far without success (Williamson, unpublished results). There is, of course,

always the possibility that JSRV contains a novel oncogene, but this aspect of JSRV did not form part of the present study. It is hoped that once the JSRV genome is cloned or the virus is produced in culture, attempts to identify the oncogene will be resumed. It is also very likely that BRV possesses an oncogene; whether it is the same as that of JSRV remains to be investigated.

1.10 MAJOR AIMS OF THIS STUDY

The primary aim of this study was to compare JSRV with, and differentiate it from, two other retroviruses associated with the JS disease complex, namely, MVV (SA-OMV I) and BRV.

Thus, the initial step was to use the purification protocol developed in the author's M.Sc. thesis, to purify the JSRV so that its polypeptide composition, biochemical, immunological and morphological characteristics could be identified. Following this, and dependant on it, was the development of techniques whereby the JSRV could be compared with, and distinguished from, other viruses. A subsidiary objective was to produce monoclonal and polyclonal antibodies against the purified JSRV, so that they could be used in the immunological characterization of JS, as well as in the development of JS-specific diagnostic and research tools (see Chapter 3).

Since MVV (SA-OMV I) was often found together with JSRV in JS - infected lungs, it was thought important to develop techniques so that the two viruses could be distinguished from each other. As

both viruses were present in the same lung wash pellet and had similar physicochemical characteristics it was necessary to develop a purification method whereby they could be separated from each other. This was achieved and is discussed in chapter 4.

Furthermore, a study was made of a novel retrovirus (BRV), suspected of playing some role in JS (see Chapter 5). This study was undertaken to elucidate whether it was identical to JSRV or merely a related virus. The techniques developed for JSRV (see Chapter 3) made it possible to study BRV and compare its biochemical, immunological and morphological characteristics with those of JSRV and MVV.

Finally, in chapter 6, the characteristics of all three viruses are collated and their possible involvement in the JS disease complex speculated upon.

It is important to note that research on JS is an ongoing process, which is as yet still in the early stages, where researchers in the same department are working very closely on the various aspects of the disease. By necessity, the biochemical, immunological, morphological and cloning studies frequently overlap. Where this has occurred, such as with the transmission electron microscopic observations and with the cloning of the JSRV cDNA, the colleagues involved have been duly acknowledged.

CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

In this chapter the techniques used to study, assay and purify JSRV, MVV and BRV are presented. The methods have been combined into one chapter, as the techniques used to study, monitor and purify all three viruses were very much the same. It should, however, be emphasized that purification of retroviruses from an in vivo source, such as JSRV from sheep lungs, required additional purification techniques to those used for retroviruses produced in vitro, such as BRV. Furthermore, virus purified from an in vivo source is rarely as clean and always less concentrated than virus produced in vitro.

The viruses were mainly monitored using the reverse transcriptase assay. In the case of JSRV and MVV magnesium was used, whereas manganese was used for BRV. The purity achieved was assessed by SDS-PAGE, as well as transmission electron microscopy. In addition, indirect immune overlay techniques were used to confirm the presence of viral antigen. These techniques were also used to confirm the specificity of the monoclonal and polyclonal antibodies made against viral preparations and to develop assays, which were used to examine the relationship between the three viruses.

2.2 TITRATION ASSAYS

A major limitation in jaagsiekte research is that there is no accurate technique which quantitatively assays solely for jaagsiekte retrovirus (JSRV) during a purification. Nevertheless, two titration assays were used to provide a semi-quantitative indication of virus concentration. These are the RDP-assay, which measures viral reverse transcriptase enzyme activity (RDP), and an enzyme-linked immunosorbent assay (ELISA), which measures viral antigen. The RDP-assay was a great breakthrough in retrovirus research and has for some time been the only way to detect the presence of JSRV. Although the RDP-assay only measures RDP enzyme activity and does not indicate whether the virus is intact or not, it provides a semi-quantitative measure which is useful during a purification. The term JSRV-RDP has been used in certain cases to emphasize that it is the enzyme that is measured and not necessarily the whole virus.

2.2.1 RNA dependant DNA polymerase assay (RDP-assay)

All virus concentrations were estimated by means of a standard RDP-assay (Verwoerd et al., 1983). This measures the incorporation of ³H-thymidine triphosphate into DNA by the viral reverse transcriptase with poly(A).(dT)₁₀ (Boehringer Mannheim Biochemica) as an artificial template-primer. Assay buffer (20 mM Tris-HCl (pH 8,3) + 0,33 mM EDTA) was used to dissolve the material to be tested. Samples (20 ul) were added to 55 ul of an assay mix to give final concentrations of 14,7 mM Tris, 0,243 mM EDTA, 5 mM MgCl₂ (MgCl₂ was replaced with MnCl₂ for the manganese dependant

retroviruses), 0,24% (w/v) Triton X-100, 18 mM KCl, 0,3 mM GTP, 14,5 mM DTT, 1,52 μ m³ H-TTP (40-60 Ci/mM) and 5,25 μ g of template-primer per assay. After incubation at 37 C for 20 min, the reaction was terminated by spotting onto DEAE cellulose filter discs (Whatman DE 81). These were dried, rinsed 6 times with freshly prepared 5%(w/v) Na HPO₂ solution, twice with distilled water and finally once in absolute alcohol. The discs were dried and counted in 5 ml Beckman Ready-Solv EP scintillation cocktail, using a Beckman LS 9 000 scintillation counter.

2.2.2 Enzyme linked immunosorbent assay (ELISA)

2.2.2.1 Reagents

Source of antisera and conjugates: Mouse monoclonal antibodies were prepared as described in 2.9. They were used undiluted if taken directly from hybridoma culture supernatants. If ascites fluid or concentrated culture supernatant was used, the monoclonal antibodies were diluted as described in the relevant figure legends. Peroxidase conjugated rabbit anti-mouse immunoglobulins were obtained from Dakopatts, Copenhagen, Denmark.

Peroxidase substrate: The peroxidase substrate consisted of 0,6% O-phenylenediamine with 0,3% hydrogen peroxide in a 0,05 M phosphate-citrate buffer, pH 5,0.

2.2.2.2 Method

A modification of the indirect ELISA technique (Voller, Bidwell

and Bartlett, 1976) was used to screen and isolate the JSRV-specific hybridomas. JS and normal lung (NL) antigens were bound to microtitre plates (Nunc-Immuno Plate I.96F, Denmark) at 1:800 dilutions in 0,05 M carbonate buffer (pH 9,6) overnight at 4 °C. The plates were washed three times with 500 ml of 1 mM Na-phosphate buffer (pH 7,2), containing 0,05% (v/v) Tween 20. A specially constructed washing shower (Conradie, Vorster and Kirk, 1981) was used to wash the plates. The plates were blocked with 10% horse serum /phosphate buffered saline (PBS), for 1 h at room temperature. Hybridoma supernatant (50 ul) was bound directly or, if concentrated culture supernatant or ascites fluid was used, it was diluted as described in the legend to the relevant figure or plate. Blocking buffer was used to dilute the antibodies. After a 2 h incubation at 37 °C the plates were washed as above. They were then incubated with the relevant conjugated anti-mouse immunoglobulin antiserum, diluted at 1:400 with 10% horse serum/PBS. After a 1 h incubation at 37 °C the plates were again washed as above. After the wells were shaken dry, 100 ul of the peroxidase substrate was dispensed into each well. The reaction was stopped after 10 min with 50 ul 2N H₂SO₄ in each well and the absorbance read at 492 nm (the maximum absorbance for this chromophore) in a Titertek Multiscan spectrophotometer.

2.2.3 Dot blot assay

The dot blot assay combines features of the standard ELISA and the Western immune blot technique. It was used to determine why the monoclonal antibodies did not react with JS antigen when it was

separated on SDS-PAGE. Once the antigen was bound to the nitrocellulose the procedures were the same as described for the immune blot technique (see 2.8.2).

The reagents used for this technique are the same as for the immune blot (see 2.8.2.1), the only difference being the blocking buffer. For the dot blot 10% horse serum/PBS was used instead of 50% horse serum.

2.2.3.1 Method

A Bio-Dot microfiltration apparatus from Bio-Rad Laboratories, Richmond, USA, was used to bind the JS antigen to the nitrocellulose. Briefly a sheet of nitrocellulose, the size of the apparatus, was incubated in PBS for 10 min. The nitrocellulose sheet was placed into the microfiltration apparatus following the supplier's instructions. After washing the wells, by drawing PBS through by suction, the antigen (100 ul) was pipetted into the wells. The sample was allowed to bind using the vacuum or allowed to filter through passively. If the latter approach was adopted, binding was complete after 20 - 30 min. This approach was preferred. The wells were then washed several times with PBS drawn through by suction or alternatively, the nitrocellulose was removed from the apparatus, marked, washed and blocked with 10% horse serum for 15 min and developed as described for the immunoblot technique (see 2.8.2)

2.2.4 Protein determination

Protein concentration was determined by means of the Peterson (1977) modification of the Lowry, Rosebrough, Farr and Randall (1951) method.

2.2.4.1 Reagents

Stock reagents:

Copper tartrate carbonate (CTC): Copper-sulphate (pentahydrate) (Merck, Schuchardt and Co.) (0,1 g), potassium tartrate (Merck, Schuchardt and Co.) (0,2 g) and sodium carbonate (Merck, Schuchardt and Co.) (10 g) was dissolved in deionized H_2O and made up to 100 ml.

20% Sodium dodecyl sulphate (SDS): SDS (Merck, Schuchardt and Co.) (20 g) was dissolved in deionized H_2O and made up to 100 ml.

Sodium hydroxide: 0,8 N.

Folin-Ciocalteu phenol reagent: This solution was obtained from Merck, Schuchardt and Co.

Working solutions:

Reagent A: Equal parts of stock CTC, NaOH, SDS and deionized water were mixed together (shelf-life 2 weeks).

Reagent B: Five parts deionized water were added to 1 part Folin-Ciocalteu phenol reagent. This solution was made up immediately before use.

Standard: Bovine serum albumin (BSA): BSA Fraction V was obtained from Miles Laboratories (Pty) Ltd, South Africa. Aliquots (1 ml) of 1 mg/ml (BSA/H₂O) were stored at -70 °C. Once thawed, samples were kept refrigerated for up to 5 days.

2.2.4.2 Method

Samples containing between 1 and 100 ug of protein were made up to 1 ml with deionized water. Reagent A (1 ml) was added, the solutions mixed and allowed to stand for 10 min at room temperature. Reagent B (0,5 ml) was added and the solutions mixed. After 30 min the absorbance of each mixture was read at 750 nm. Three replicates of 20 and 100 ul of BSA-standard (1 mg/ml) were used to determine the standard values used in the calculation of the unknown protein concentration, using the following equation:

$$\text{Protein (ug)} = (I \times A_{750}^S)$$

where

$$S = \log (h/l) / (A_{750}^h / A_{750}^l)$$

$$I = \text{antilog} [(\log h) / S - \log A_{750}^h]$$

In this equation h and l refer to the concentrations of the high and low protein standards, respectively, and A_{750}^h and A_{750}^l to their corresponding absorbance values at 750 nm. If the unknown protein's absorbance fell outside the range of the standards, one of the following alternatives were taken: (a) additional standards were made, or (b) the unknown protein was diluted until its absorbance range fell within the calibrated range.

2.2.5 Estimation of RNA concentration using an ethidium bromide/agarose method

This is a simple and effective method to determine the RNA concentrations of very small volumes.

A 1% agarose (Miles Laboratories) solution was made by dissolving 1g in 100 ml TE buffer (see Appendix 1). The mixture was heated in a microwave oven to dissolve the agarose. Before the agarose set, 15 μ l ethidium bromide (10 mg/ml) was added and the mixture poured into petri dishes. These could be stored in the dark for a few days, or used immediately once the agarose had cooled to room temperature.

To determine the RNA concentration of an unknown sample, a standard reference series was made by spotting 2 μ l aliquots of known RNA concentrations, ranging between 0,02 μ g/2 μ l to 0,1 μ g/2 μ l, in a row on the agarose plate. Two dilutions of the unknown, also 2 μ l, were spotted below the standards. After 15 to 30 min the plate was inverted and observed on a UV light box. The intensity of the unknown was compared to the standards, which enabled a fairly accurate estimation of the RNA concentration to be made.

2.3 SOURCE OF MATERIALS

2.3.1 JSRV-isolation

The jaagsiekte material used in this study was isolated from the lungs of jaagsiekte infected sheep produced by serial inoculation

of semi-purified virus, originally isolated from a field jaagsiekte case into the trachea of newborn lambs. An inoculation of 1 million RDP-units was normally sufficient to produce clinical symptoms (dispnoea) in 6-8 weeks. When the disease was advanced the lambs were slaughtered; their lungs were removed and immediately rinsed in 1,5 litres of ice-cold PBS (see Appendix 1). All further steps were carried out at 4 °C. The lungs were rinsed by pouring 500 ml PBS into the trachea and massaging the lungs gently but thoroughly. This was repeated three to four times. The lung rinse material was clarified by low speed centrifugation (10 000 x g; 30 min; 4 °C) to remove cells and cell debris.

The clarified lung rinse material was pelleted in a Beckman Ti 15 batch rotor for 2,5 h at 30 000 rpm. The supernatant was aspirated off, leaving the pellet, which was scraped off the rotor wall with a rubber policeman. The pellet was resuspended in 0,5 M Na-phosphate buffer (pH 7,2) containing 0,5 mM DTT, giving a final volume of 15 ml. The resuspended pellets were stored at -70 °C.

2.3.2 SA - OMVV I - isolation

Sheep lentivirus was grown in secondary cultures of sheep choroid plexus cells. These cells were infected with untreated jaagsiekte lung rinse pellets. Approximately two weeks after the infection, the culture medium was collected and clarified by low speed centrifugation (3 000 x g; 10 min; 4 °C) in a Beckman JS-7,5 swinging bucket rotor. The virus was pelleted from the supernatant (55 000 x g; 90 min; 4 °C) in a Beckman Type 30 rotor. The pellet was resuspended in assay buffer (see 2.7.1) at 1% of the original

concentration and stored at ^o-70 C for later use.

2.3.3 BRV - isolation

The original isolation of BRV was made by Dr Barnard of the Virology section, Onderstepoort. He kindly provided us with the transformed calf foetal thyroid (CFTH) culture which is the source of BRV.

Cells were cultured in Eagle's medium supplemented with 10% bovine serum, penicillin (1000 000 u/l) (Novo) and streptomycin (100 mg/l) in roller bottles (100 ml/roller). On day 7 the medium was harvested and clarified (5 000 rpm; 10 min; 4 C) before pelleting in a Beckman Type 30 rotor (55 000 x g; 90 min; 4 C). The pellet was resuspended in TNE buffer (see Appendix I) (1ml/roller) and stored at ^o-70 C.

2.3.3.1 Radiolabelling of BRV

The medium was removed from 7 day old BRV cultures and replaced with 10 ml Eagle's medium supplemented with 10% dialysed FCS containing 700 ul [5,6- ³H]-Uridine (40 Ci/mmol) (Amersham International plc). The cells were incubated for a further 48 h after which the medium was clarified and concentrated as above. The labelled virus was applied directly onto isopycnic gradients and centrifuged as described (2.5.1.1). Samples from the gradient fractions (100 ul) were added to 5 ml aqueous scintillation fluid

(see Appendix I) and counted in a Beckman LS 9 000 scintillation counter. When the cells were labelled with ³²P-orthophosphate or ³⁵S-methionine, the Eagle's medium was phosphate or methionine free. Otherwise the method was the same.

2.4 FLUOROCARBON (FREON) EXTRACTION

Freon 113 is a synonym of trifluorotrichloroethane, a non-polar lipid solvent. It is believed to manifest a strong protein-denaturing effect at the solvent-water interphase. Since it does not denature conjugated proteins it is widely utilized in the purification of viruses from crude suspensions containing contaminating components susceptible to this type of denaturation. In general, only non-lipid viruses can be purified using fluorocarbon, since the lipid viruses would be inactivated (Philipson, 1967).

The procedure is fairly straightforward: Two volumes of cold fluorocarbon (Freon 113, Du Pont, Wilmington, Del.) were mixed with either thawed out stored pellets or fresh material. The mixture was homogenized for 5 min at 10 000 rpm in a Virtis 60K homogenizer. If the lung rinse material was fairly clean, the mixture was shaken by hand for 5 min instead of homogenizing. After phase separation by centrifugation (17 600 x g; 10 min; 4 °C) the supernatant was collected and the interphase extracted once by shaking for 3 min with a small amount of 0,01 M Na-phosphate buffer (pH 7,2). The aqueous layers were combined for further purification.

2.5 CENTRIFUGATION

2.5.1 Density gradient centrifugation

The rate at which a particle sediments in a centrifugal field depends on its size, shape and density and also on the viscosity and density of the suspending medium. If regions of a density gradient have densities corresponding to those of the particles being separated, then the separation obtained after centrifugation to equilibrium will depend on the densities of the particles. This is called isopycnic or equilibrium centrifugation. Alternatively, the particles may be separated on size and shape and not on density. This is referred to as rate-zonal separation. Both isopycnic and rate-zonal separations were used to purify the viruses from associated non-viral material.

2.5.1.1 Isopycnic gradients

Isopycnic centrifugations were carried out in 20 - 50% sucrose gradients, unless otherwise stated. Sucrose solutions were made up in buffer B (see Appendix I) and the percentage sucrose in each solution was measured using a Bausch and Lomb refractometer. A layer of 65% (w/v) sucrose solution (1 ml) was always layered onto the bottom of the tube before the gradient was added. The gradient was made using a Beckman density gradient former. The sample was layered onto the gradient and centrifuged ($85\,000 \times g$; 16 h; 4 °C) using a Beckman SW 27 or SW 41 rotor. Fractions were collected from the bottom of the tube, using a long needle attached to a peristaltic pump, and their densities determined refracto-

metrically. Virus was localized by testing fractions for RDP-activity and/or samples were concentrated (see 2.7) and separated on SDS-PAGE (see Methods 2.8).

2.5.1.2 Percoll gradients

Percoll gradients were used to concentrate or purify Freon extracted jaagsiekte lung rinse material. Percoll is a product of Pharmacia, Uppsala, Sweden and consists of colloidal silica particles of 15 - 30 nm diameter, which have been coated with polyvinylpyrrolidone and are completely non-toxic to cells. On centrifugation it generates self-forming gradients in the density range 1,0 to 1,13 g/ml. Percoll has a low osmolarity (20 M Os/kg H₂O), is inert and has many advantages over other density gradient media such as sucrose, glycerol and Ficoll, in which the virus is exposed to high osmolarities which tend to degrade the viral particle (Pertoft, Rubin and Kjellen, 1977). The aqueous phase obtained after Freon extraction (9 ml) was well mixed with Percoll (3 ml) in a capped tube and centrifuged (40 000 x g; 20 min; 4 °C) in a Beckman Type 65 rotor. Two visible bands were always obtained: an upper band in the top 7 ml containing 20% of the virus and a lower band in the remaining 4 ml containing 80% of the virus. The lower band (4 ml) was carefully collected and further purified.

2.5.1.3 Centrifugation in dissociating media

Guanidinium chloride treatment: A 4 M guanidinium chloride solu-

tion in 15% sucrose was layered onto a sucrose gradient. Viral material was layered onto the guanidinium chloride layer and allowed to sediment through to isopycnic equilibrium. Centrifugation ($80\,000 \times g$; 16 h; 4 °C) was in either a Beckman SW 41 or a SW 27 rotor.

2.6 GEL CHROMATOGRAPHY

Degassed Sephacryl S1000 superfine gel (Pharmacia, Uppsala AB, Sweden) (100 ml) was poured into a column (Pharmacia Kl6 x 40 cm) using an attached reservoir. The gel was packed by passing 0,01 M Na-phosphate buffer (pH 7,2) containing 10 mM EDTA and 10 mM DTT through the gel at a rate of 50 ml/h. After packing, the total volume was approximately 65 ml. The packed column was equilibrated with the same buffer at 4 °C and a flow rate of 40 ml/h. The sample (3-4 ml) was applied to the column and eluted with the same buffer at a flow rate of 40 ml/h at 4 °C. The effluent was monitored at 280 nm, using an LKB Uvicord II, and collected in 1 ml fractions. Virus concentration was measured in each fraction by assaying RDP-activity. Virus was concentrated by pelleting, vacuum dialysis or freeze-drying (see 2.7).

2.6.1 Affinity chromatography

Affinity chromatography is a simple procedure which can provide dramatic purification of biologically active molecules. It has several advantages over alternative separation methods: simplicity, speed, yield, high purification and concentration. There are two basic requirements in affinity chromatography:

- 1- A suitable insoluble derivative of the ligand and
- 2- An effective means of dissociating the complex formed.

In the studies reported in this thesis, cyanogen bromide coupled to Sepharose 4B (Pharmacia) was used to make the insoluble derivative of the ligand, in this case a monoclonal antibody. Potassium thiocyanate was used to dissociate the complex formed.

2.6.1.1 Reagents

CnBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden): One gram per 10 mg antibody.

Coupling buffer: 0,1 M NaHCO_3 (pH 8,3) containing 0,5 M NaCl.

Washing buffers: 0,1 M acetate buffer (pH 4) containing 1 M NaCl; 0,1 M borate buffer (pH 8) containing 1 M NaCl; phosphate buffered saline (PBS).

Blocking buffer: 1 M Ethanolamine adjusted to pH 8 with HCl.

Eluent: 3 M Potassium thiocyanate containing 0,5 M ammonia.

2.6.1.2 Method

Coupling: The method used was very similar to that described in the supplier's instructions. Briefly, 1 g CnBr-activated Sepharose 4B was washed with 200 ml 1 mM HCl over a period of 1 h using a sintered glass filter and a low vacuum. This was to remove the dextran and lactose used to stabilize the support. Ten milligrams of monoclonal antibody, purified using Protein A (see 2.9.3) in

coupling buffer, was mixed with the washed gel in a stoppered test tube. This mixture was rotated, end over end, overnight at 4 °C. The following day the gel was resuspended in coupling buffer (5ml) and packed into a used PD 10 column (Pharmacia, Uppsala, Sweden). The gel was washed with coupling buffer (3 X 15 ml) and any remaining active groups were blocked with blocking buffer for 2 h. The gel was washed with 5 ml acetate buffer (pH 4), followed by 5 ml borate buffer (pH 8). This washing procedure was repeated twice more.

Column application: The sample to be purified was run into the column at 10 ml/h. The eluant was collected and passed through a second time. The column was washed with PBS until the absorbance at 280 nm reached base line. The bound antigen was eluted with 3 M KSCN containing 0,5 M ammonia. Fractions (5 ml) were collected and were either dialysed against PBS or precipitated with SDS/KCl (see 2.7.2) to concentrate the proteins for SDS-PAGE.

2.7 SAMPLE CONCENTRATION

The final purification technique used in purifying the virus determined which method was used to concentrate the sample.

2.7.1 Pelleting

2.7.1.1 Macro scale

Samples containing sucrose were diluted 4 times with 0,05 M Na-phosphate buffer (pH 7,2) and pelleted (40 000 x g; 60 min; 4 °C)

in a Beckman Type 65 rotor. Samples collected during gel chromatography were either pelleted as above or freeze-dried (see 2.7.3)

2.7.1.2 Micro scale

Virus-containing gradient fractions that were to be analysed on SDS-PAGE were concentrated on a small scale. This had the advantage of being faster than the approach used in 2.7.1.1. The sample (400 μ l) was diluted with 1,0 ml TNE or PBS in an Eppendorf tube. The mixture was inverted three times and centrifuged for 40 min in a Sigma mark 2 microfuge (10 000 x g; 4 °C). The supernatant was poured off, the pellet dissolved in 60 μ l PSB and after heating, applied directly to SDS-PAGE (see 2.8.1).

2.7.2 SDS/KCl Precipitation

In many cases when the protein content of a sample was to be tested on SDS-PAGE, it was useful to have a quick concentration method. The most common application was to column eluants and sucrose gradient fractions. The method was very simple and only required 3 M KCl and 5% SDS solutions. The sample to be concentrated (300 μ l) was transferred to an Eppendorf tube and 30 μ l SDS was added. The tube was inverted a few times before 30 μ l 3 M KCl was added. After a few more inversions the sample was centrifuged in a microfuge for 2 min. The supernatant was discarded and the pellet dissolved in 60 μ l PSB (see 2.8.1.1) before boiling and application to SDS-PAGE. The volumes were scaled up for more dilute samples.

2.7.3 Freeze-drying

Fractions obtained during gel chromatography were concentrated using a CX 10 Millipore filter under vacuum, to reduce the volume to approximately 1-2 ml. The concentrated samples were then freeze-dried. The pellets and the freeze-dried samples were dissolved in 0,05 M Na-phosphate buffer (pH 7,2) to yield a solution approximately 200 times more concentrated than the starting solution. Ten to fifteen microlitres of the concentrated solution were analysed by SDS-PAGE.

2.8 GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) has become a popular method for analysing proteins, but it can provide information only on the size and the amount of these molecules. The unambiguous identification of a protein requires additional tests that respond to the amino acid sequence. Nevertheless, SDS-PAGE provides a very useful, rapid and straightforward method of analysing the polypeptide composition in a sample. For this reason it was the initial method used to identify viral polypeptides. The usefulness of SDS-PAGE depends on the ability of the detergent, sodium dodecyl sulphate (SDS), to interact with and denature a wide variety of proteins in a similar manner (Reynolds and Tanford, 1970). Native proteins, having different charge, size and shape characteristics, are converted upon reduction and SDS binding to SDS-protein complexes of their constituent polypeptide chains. These SDS-protein complexes

have a constant charge per unit mass and identical conformations, therefore separating during electrophoresis according to the protein's subunit molecular weight(-s). However, the larger complexes, although they have a larger negative charge, are retarded by the gel matrix and therefore move slower than the smaller polypeptides which move in front. In SDS-PAGE, protein molecular weights can be determined by comparing the electrophoretic mobilities of unknown proteins with the electrophoretic mobilities of standard proteins of known molecular weight.

A very useful extension of gel electrophoresis is immune blotting (Towbin, Staehelin and Gordon, 1979). In this method proteins which have been separated by SDS-PAGE are electrophoretically transferred to an insoluble matrix, such as chemically reactive cellulose, or more conveniently, commercially available nitrocellulose sheets. This transfer yields a faithful replica of the original gel pattern. The immobilized proteins on the replica are readily accessible to antibodies or other specific probes, eg. lectins. This makes possible the in situ localization and characterization of individual polypeptides in complex mixtures.

2.8.1 SDS-PAGE

2.8.1.1 Reagents

(All reagents used were from Merck, Schuchardt and Co., unless otherwise stated.)

Acrylamide/Bisacrylamide: Acrylamide (30 g) and bisacrylamide (0,8 g) were dissolved in 50 ml deionized H₂O and made up to 100 ml.

Separating gel buffer: Trizma base (Sigma Chemical Company) (36,6 g) and SDS (0,8 g) were dissolved in 150 ml deionized H₂O, adjusted to pH 8,8 with HCl and made up to 200 ml.

Stacking gel buffer: Trizma base (Sigma Chemical Company) (12,1 g) and SDS (0,8 g) were dissolved in 150 ml deionized H₂O, adjusted to pH 6,8 with HCl and made up to 200 ml.

TEMED: N,N,N',N'-tetramethylethylenediamine was stored at 4 °C.

Ammonium persulphate: This solution was freshly made up by dissolving 0,1 g ammonium persulphate in 1,5 ml deionized H₂O.

Stock reservoir buffer: A ten times stock reservoir buffer was prepared by dissolving Trizma base (Sigma Chemical Company) (126,4 g) and glycine (79,8 g) in 600 ml deionized H₂O, adjusting to pH 8,9 with HCl and making up to 1 litre.

Reservoir buffer: The reservoir buffer was prepared by diluting 100 ml of stock to 990 ml and adding 10 ml 10% SDS. The pH should be 8,9.

Protein solubilizing buffer (PSB): PSB was prepared by mixing together 5,5 ml stacking gel buffer (pH 6,8), 2,0 ml 10% SDS, 1,0 ml glycerol, 0,5 ml 2-mercaptoethanol and 0,01% (w/v) bromophenol blue in water.

Serva Blau G staining solution: The staining solution was made up by dissolving 1 g Serva Blau G (Serva Feinbiochemica and Co.) in a mixture of 300 ml methanol, 50 ml glacial acetic acid and 200 ml deionized H₂O.

Destaining solution: The destaining solution was made up by diluting 100 ml glacial acetic acid in 2 litres hot tap water.

Molecular weight calibration markers: A low molecular weight calibration kit containing 6 proteins with molecular weights of 94 000, 68 000, 43 000, 30 000, 20 000 and 14 000 dalton, respectively, was obtained from Pharmacia, Uppsala AB, Sweden. The freeze-dried proteins were dissolved in 800 μ l reservoir buffer and 10 μ l aliquots were stored at -70°C .

12,5% Separating gel: To make a 12,5% separating gel, 12,5 ml acrylamide/bis (30%; 0,8%) stock, 7,5 ml separating gel buffer, 9,8 ml deionized H_2O and 0,0125 ml TEMED were added into a 250 ml Ehrlenmeyer flask. Immediately prior to pouring, 0,188 ml ammonium persulphate (stock) was added. To alter the percentage gel, the volume of the acrylamide/bis was adjusted and the volume of H_2O corrected to maintain the same total volume, viz for a 10% separating gel, 10 ml acrylamide/bis was used and (9,8 + 2,5 ml) i.e. 12,3 ml H_2O .

5% Stacking gel: The stacking gel solution was prepared by mixing together 2,0 ml acrylamide/bis (30%; 0,8%) stock, 3,0 ml stacking gel buffer, 4,0 ml deionized H_2O and 0,015 ml TEMED in a 100 ml Ehrlenmeyer flask. Immediately prior to pouring the stacking gel, 3,0 ml ammonium persulphate (0,5 ml stock) + 11,4 ml H_2O was added.

2.8.1.2 Method

One-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis of "viral proteins" was carried out in a 0,1% SDS-containing 12,5 or 10% polyacrylamide gel with a 5% stacking gel, according to the method of Laemmli (1970). Briefly, two clean glass plates were separated by three 1 mm thick perspex spacers. These spacers were sealed with a 2% agarose solution. The gel solution was poured between the plates until a mark 4 cm from the top was reached. About 1 ml water was gently layered onto the top of the separating gel. After 45 min the water layer was poured off, the stacking solution poured onto the set separating gel and the required comb inserted between the plates. After the stacking gel had set (ca. 15 min) the comb and the bottom spacer were removed under water and the gel assembled in an apparatus constructed in this laboratory. The top and bottom reservoirs were then filled with reservoir buffer.

The samples to be analysed (10-20 μ l) were dissociated by adding an equal volume of PSB and heating at 100 $^{\circ}$ C for 3 min. A sample containing low molecular weight markers (10 μ l) was normally included in each run. Samples were dispensed into the wells with a graduated microsyringe (Hamilton Corp., California) The syringe was rinsed 4 times with an electrophoretic buffer followed by 4 rinses with deionized water between samples.

Electrophoresis was carried out at 10,2 V/cm for 12 h, unless otherwise stated. The separated protein bands were stained in

staining solution for 30 min and destained in 5% acetic acid overnight at room temperature, or for 3 h at 60 C. After destaining the gel was placed on a viewing box and photographed with a Type 665 Polaroid film. If the separated proteins were to be analysed by the immunoblot technique (see 2.8.2), the staining step was omitted.

2.8.1.3 Molecular weight determination

For molecular weight determinations, a calibration curve was constructed by plotting R_m versus \log (molecular weight) of the standard proteins. The relative mobility (R_m) for each of the proteins was calculated as follows:

$$R_m = \frac{\text{distance protein has migrated from origin}}{\text{distance from origin to reference point}}$$

The measurements were taken from a photograph of the gel, using the lowest (14 000 dalton) standard protein as the reference point. Plate 2b shows a calibration curve which was constructed using the R_m values of the marker proteins run in Plate 2a. The curve was used to determine the molecular weights of the JSRV polypeptides in Plate 2a.

2.8.2 Immunoblot technique

2.8.2.1 Reagents

Washing buffer: The washing buffer was prepared by dissolving 42 g NaCl and 0,5 ml Tween 20 (Bio-Rad Laboratories) in 2 litres phosphate buffered saline (PBS).

Blocking buffer: A 50% blocking solution was prepared by diluting 500 ml horse serum with 500 ml PBS.

Primary sera: Rabbit anti-JSRV serum was used at a 1:100 dilution. All sera were diluted with blocking buffer.

Sheep anti-MVV (Maedi-Visna virus) serum was prepared by the Department of Molecular Biology, VRI Onderstepoort and used at a 1:50 dilution.

Goat anti-MPMV (Mason-Pfizer monkey virus) p27 serum was obtained from the National Cancer Institute, Bethesda, USA and used at a 1:400 dilution.

Horseradish peroxidase-conjugated antibodies against primary serum (peroxidase conjugates): Rabbit anti-sheep IgG peroxidase conjugate was obtained from Dakopatts, Copenhagen, Denmark and was used at a 1:400 dilution.

Horseradish peroxidase substrate solution: The peroxidase substrate was freshly prepared by dissolving 4-chloro-1-naphtol (Bio-Rad Laboratories) (0,06 g) in 20 ml ice-cold methanol. The substrate solution was mixed with 100 ml PBS and 60 μ l H_2O_2 immediately before use.

Amido black staining solution: The staining solution was prepared by dissolving amido black powder (Merck, Schuchardt and Co.) (0,1 g) in a mixture of 45 ml methanol, 10 ml acetic acid and 45 ml deionized H_2O .

Amido black destaining solution: The destaining solution consisted of 450 ml methanol, 40 ml H_2O and 10 ml glacial acetic acid.

Transfer buffer: Glycine (192 mM) (Merck, Schuchardt and Co.) (57,46 g) and Trizma base (25 mM) (Merck, Schuchardt and Co.) (12,11 g) were dissolved in 2 litres deionized water and adjusted to pH 8,3 with HCl. Methanol (800 ml) was added and the solution made up to 4 litres with deionized water.

2.8.2.2 Method

SDS-protein complexes, separated as outlined above (2.8.1.2), were transferred to nitrocellulose sheets using an apparatus made by Bio-Rad Laboratories. The SDS-gel was placed onto an equal sized nitrocellulose sheet (Bio-Rad Laboratories) which had been equilibrated in transfer buffer for 10 min. The gel and nitrocellulose sheet were sandwiched between layers of filter paper (Whatman No 1) and Scotchbrite pads (3M Co. Ltd.). The sandwich was enclosed in a hinged perspex holder and inserted into the transfer chamber, ensuring that the nitrocellulose sheet was anodal to the gel. The transfer chamber was filled with transfer buffer and transfer was carried out at 0,45 amps for 4 h at 4 C. After transfer the gel was stained in Serva Blau G staining solution (see 2.8.1.1) for 30 min and destained in 5% acetic acid (see 2.8.1.1) to assess the efficiency of the protein transfer. For direct visualization of transferred proteins, the nitrocellulose sheet was stained for 1-2 min with amido black (see 2.8.2.1), followed by rapid destaining with amido black destaining solution (see 2.8.1.2).

For immunological detection of transferred proteins, the nitro-

cellulose was cut into the desired strips and blocked in 50% horse serum/PBS (v/v) for 1 h with gentle rocking. The blocking buffer saturated all remaining protein binding sites on the nitrocellulose. The nitrocellulose strips were then incubated with the relevant primary antiserum and gently rocked for 2-16 h. Excess and unbound primary antiserum was washed off with washing buffer (3 x 10 min) and the strips were rinsed twice with deionized water. Washed strips were incubated for 1 h in the relevant horseradish peroxidase-conjugated antibodies directed against the primary antibody. Excess conjugate was washed and rinsed off as described earlier. The strips were added to a freshly prepared peroxidase substrate solution. When the bands were clearly visible (after 10 - 15 min) the reaction was terminated by immersing the strips into deionized water for 10 min. The strips were dried between filter paper, photographed and stored in the dark.

2.9 MONOCLONAL ANTIBODY PRODUCTION

2.9.1 Introduction

Monoclonal antibodies (MAbs) have many uses, two of which are as diagnostic and research tools. An advantage that MAbs have over polyclonal antibodies, which is most relevant in jaagsiekte research, is that the immunogen does not have to be pure to obtain a pure antibody. In the study reported in this thesis attempts to produce and isolate MAbs that could be used as jaagsiekte-specific diagnostic and research tools, are described.

To produce mouse MAbs, a mouse is immunized with a preparation

that contains the antigen(s) against which antibodies are required. After a few immunizations the spleen contains a bank of antibody producing cells against the immunogen. These cells are then immortalized by fusion with myeloma tumour cells with the aid of polyethyleneglycol. The resulting hybrid cell, or hybridoma, exhibits characteristics of both parent cells, is immortal and secretes antibodies of a specificity determined by the immune donor. However, because the efficiency of the fusion is relatively low, a selection method is required otherwise the immortal unfused cells would outgrow the fused cells. In the fusions performed, immortal cells (SP2/0), deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT-ase), were used. When these immortal cells are grown in aminopterin they die, but the normal spleen cells do not. Therefore when the immortal cells are fused with normal spleen cells, the salvage pathway of the spleen cells comes into operation and the hybridomas survive.

By a selection method, normally an ELISA or radioimmunoassay (RIA), it is possible to localize those hybridomas that are producing antibodies specific for the antigen used in the screening method. By a method of limiting dilution it is then possible to isolate a single clone (monoclonal) and by growing this up, have identical cells producing antibodies of one specificity. Scaling up can be done in vivo by injecting the hybridomas into the peritoneum of a mouse compatible with the immortal cells used in the fusion. These hybridoma cells then produce very high concentrations of antibody in the form of ascites fluid.

2.9.2 Methods

The methods used to produce mouse hybridomas were essentially as described in Johnstone and Thorpe (1982).

2.9.2.1 Immunization

Five balb/c mice were immunized with purified JSRV antigen (see 3.2.1.1). The antigen was prepared by mixing 100 μ l (50 μ g) JSRV with 100 μ l Freund's complete adjuvant (Difco Laboratories) per mouse. The antigen was injected subcutaneously at two sites into mice six weeks old (day 0). On days 14 and 21 the mice were each injected with 25 μ g JSRV antigen (50 μ l JSRV and 50 μ l Freund's incomplete adjuvant). The mice were bled on day 35 and their titre determined using an ELISA (see 2.2.2). Six weeks later (day 77) the mouse with the highest titre was boosted with 50 μ g JSRV antigen (50 μ l JSRV in PBS) intravenously. The mouse was subsequently sacrificed (day 81) and its spleen aseptically removed.

2.9.2.2 Hybridoma and myeloma (SP2/0) culture medium

Dulbecco's Modified Eagle's Medium (DMEM) was prepared according to the formula of Naval Biosciences. The medium was supplemented with 20% foetal calf serum (FCS) (Highveld Biologicals (pty) Ltd) and 1 ml of a 0,2 M L-glutamine stock solution (stored at ^o-20 C) per 100 ml medium. Penicillin (10 000 units), Streptomycin (10 mg) and Gentamycin (1mg) were also added per 100 ml medium.

2.9.2.3 Preparation of thymocyte conditioned medium (TCM)

Ten to fourteen day old mice were sacrificed and their thymuses removed aseptically. The thymus from each mouse was transferred to a petri dish containing 5 ml DMEM and the thymocytes liberated by gently rubbing the tissue through a tea strainer. The cells were resuspended in 25 ml DMEM supplemented with 20% FCS and incubated at 37 °C for 48 h. The medium was clarified (1000 x g; 10 min) and stored at -20 °C.

2.9.2.4 Myeloma Cells

Mouse myeloma cells (SP2/0) deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRT) were kindly provided by Dr N.T. van der Walt (Department of Biochemistry, VRI, Onderstepoort). They were grown in 20% FCS/DMEM (see 2.9.2.2) at 37 °C in a 5 - 7% CO₂ atmosphere. A Forma Scientific water-jacketed incubator was used.

2.9.2.5 Fusion

The mouse with the highest antibody titre was sacrificed and its spleen aseptically removed and transferred to a petri dish containing 5 ml DMEM. Using two bent 19 gauge needles on 5 ml syringes, the spleen cells were gently squeezed out from the spleen sac. The cells were suspended in 10 ml serum-free DMEM and transferred to a 10 ml sterile conical tube. The cells were

pelleted (600 x g; 10 min) and washed again with serum-free DMEM. After the second wash the spleen cells were combined in a 30 ml Corex tube with 1×10^7 SP2/O myeloma cells which had also been washed in parallel with the spleen cells. The combined cells were washed a third time with 20 ml serum-free DMEM (600 x g; 10 min). As the myeloma cells are much larger than the spleen cells, it is important that the third wash should be done in a round bottom tube, avoiding the differential centrifugation effect which occurs in conical tubes. The supernatant was removed and the cells were resuspended in the supernatant that remained after pouring off, by gently tapping the tube.

The cells were fused by adding a total of 1 ml 40% PEG (MA Bio-products, mw 1 500) dropwise over a period of 60 seconds. The suspension was incubated for 90 seconds at 37 C, after which 5 ml serum-free DMEM was added, in a dropwise fashion, over a period of 5 min (1ml/min), with constant swirling. The suspension was left at room temperature for 10 min before transfer to 195 ml HAT-containing medium (DMEM supplemented with 20% FCS, 50% TCM (2.9.2.4) and 1×10^{-4} M) [hypoxanthine (1×10^{-4} M); aminopterin (4×10^{-7} M); thymidine (1×10^{-6} M)] (Flow Laboratories). The mixture was distributed into the wells of 96-well microtitre plates (Costar Laboratories) (20 ml/plate). The plates were incubated for four days at 37 C in 5 - 7% CO₂ before the medium was aspirated off and replaced with HT medium (DMEM supplemented with 20% FCS, 50% TCM and 1×10^{-4} M) [hypoxanthine (1×10^{-4} M); thymidine ($1,6 \times 10^{-5}$ M)] (Flow Laboratories). Thereafter the medium was replaced with DMEM containing 20% FCS at six day intervals. The wells were

monitored microscopically for colony growth and were screened for antibody production after the second week.

2.9.2.6 Cloning by limiting dilution

Cloning is the process by which an individual cell is isolated and grown into a homogeneous colony of cells. There are two methods of cloning: 1) in soft agar or 2) by limiting dilution. The latter was used. In this method cells were diluted so that they could be individually pipetted into separate containers at theoretical concentrations of 1 cell/well and 2 cells/well.

A sample (45 ul) containing hybridomas from a positive fusion well, was mixed with 5 ul of a 1% trypan blue/PBS solution. A viable cell was obtained using a Neubauer cell counting slide. The cells were then diluted in 20% FCS/DMEM supplemented with 50% thymocyte conditioned medium (see 2.9.2.3) to make two dilutions in 5 ml each of 1 cell/100 ul and 0,5 cell/100 ul, respectively. Two drops (100 ul) of each dilution was pipetted into each well of a 96-well microtitre plate (Costar). The plates were incubated in 5 - 7% CO₂ for 1 - 3 weeks. Wells were scanned under the microscope after 7 days to identify those wells with colonies. The colonies were then screened for antibody production (see 2.2).

2.9.2.7 Ascites production

Hybridomas will grow in the peritoneal cavity of animals of the same inbred strain from which the tumour cell line and spleen cells were derived and will secrete monoclonal antibodies into the

ascites fluid. With this procedure as much as 10 mg antibody/ml fluid can be recovered.

Mice were injected (intraperitoneally) with 0,5 M pristane (Sigma Chemical Co.) which suppresses killer cell activity. Seven days later 5×10^6 to 1×10^7 hybridoma cells/ 0,5 ml PBS were injected intraperitoneally using a narrow gauge needle. After a few days, when swelling of the abdomen could be observed, the ascites fluid was tapped off using a wide gauge needle. The fluid was clarified by centrifugation ($500 \times g$; 10 min) and stored at -20°C . The mice were tapped once or twice more at 2 day intervals before they died.

2.9.3 Purification of MAbs using protein A

2.9.3.1 Introduction

Protein A is a group-specific protein, synthesized by some strains of Staphylococcus aureus, that binds to the Fc region of IgG from many species. When coupled to agarose beads, protein A can be used to purify IgG. Mouse IgG is comprised of four major subclasses designated IgG1, IgG2a, IgG2b and IgG3. These subclasses differ in their ability to bind to protein A and can be separated by affinity chromatography on immobilized protein A. The bound IgG subclasses can be eluted differentially using buffers with various pH values (Ey, Prowse and Jenkins, 1978). This technique is especially useful in purifying and concentrating MAbs from ascites fluid or hybridoma culture supernatant.

2.9.3.2 Reagents

Protein A coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden):

Buffers: The following eluting buffers were made from 0,1 M citric acid by the addition of NaOH to adjust the pH: 0,1 M sodium citrate, pH 6,0; pH 4,5 and pH 3,5. Additional buffers used were 2 M Trizma base (Sigma Chemical Co.) and 0,1 M sodium phosphate (pH 8), containing 0,02% sodium azide.

2.9.3.3 Method

Protein A coupled to Sepharose 4B (1 g) was swollen with 5 ml 0,1 M phosphate buffer and packed into a previously used PD 10 column container (Pharmacia, Uppsala, Sweden). The gel was washed with 2 ml citric acid followed by 5 ml 0,1 M phosphate buffer (pH 8,0). The ascites or hybridoma culture supernatant was adjusted to pH 8,0 with 2 M Trizma base before it was loaded onto the gel at 5 ml/h. The eluant was collected and passed through a second time. The column was washed with 5 ml 0,1 M sodium phosphate buffer. IgG1 was eluted with 5 ml sodium citrate buffer (pH 6,0), IgG2a with 5 ml sodium citrate buffer (pH 4,5) and finally IgG2b with 5 ml sodium citrate buffer (pH 3,5). The latter two eluants were collected in tubes containing 2 M Trizma base (120 ul/ml eluant) so as to neutralize the low pH.

2.9.4 Characterization of monoclonal antibody subclass

2.9.4.1 Introduction

To determine the subclass of the MAb of interest, a kit from Zymed Laboratories, Inc. was used. The kit supplied a selection of affinity purified rabbit antibodies to mouse IgA(Fc); IgG1; IgG2a; IgG2b; IgG3; IgM(Fc); Kappa and Lambda light chains. The method uses an ELISA essentially as described in 2.2.2.2. A description of the method is as follows (the reagents are described in 2.2.2.1) .

2.9.4.2 Method

Antigen, against which the MAb of interest was directed, was bound overnight to 9 wells (or 18 wells for duplicate assays) in carbonate buffer. After washing (described in 2.2.2.2), the MAb (50 ul) was added undiluted to each well and incubated for 1 h at 37 C. After washing as above, 50 ul of each subclass-specific rabbit anti-mouse immunoglobulin was added to 8 out of the 9 wells or, if duplicated, 16 out of the 18. Normal serum was added to the remaining wells, as controls. After a further 1 h incubation at 37 C and subsequent washing, peroxidase conjugated goat anti-rabbit IgG(H+L) (50 ul) was added and the wells incubated for another hour at 37 C. The wells were washed and OPD peroxidase substrate (see 2.2.2.2) was added (100 ul/well). The positive wells were observed after about 20 min, the reaction was stopped with 50 ul 2 M H₂SO₄, and the plates read at 492 nm.

2.10 IMMUNOCYTOCHEMISTRY

An indirect immunoperoxidase method (Johnstone and Thorpe, 1982) was used to detect antigen related to the MAbs and polyclonal MPMV and MWV antisera in frozen lung sections and tumour cell lines.

2.10.1 Preparation of frozen lung sections

Lung tissue sections (3mm³) were cut out from normal and jaagsiekte infected lungs immediately after the lungs had been rinsed and were snap frozen in an isopentane slush. The slush was made by suspending a beaker of isopentane in liquid nitrogen for about 10 min. Each block was placed into a pre-labelled Eppendorf tube that had been pre-cooled in liquid nitrogen. The blocks were either stored under liquid nitrogen or sectioned immediately. Frozen sections were cut using a Reichert Jung Cryo-Cut II microtome and air dried on clean glass slides. The sections were stored in slide holders which were sealed in plastic bags at -20 °C until stained.

2.10.2 Preparation of formalin fixed lung sections

Tissue sections fixed with formalin have the advantage that they can be mounted in paraffin. The cell resolution obtained during this approach is better compared with frozen sections. A major disadvantage, though, is that the antigenicity of the proteins is often lost with formalin fixing.

Lung tissue sections (1 cm x 1 cm x 2 cm) were cut from the lungs and fixed in 10% formalin for a minimum period of 24 h. The fixed blocks were then dehydrated with 70% alcohol (1 h), 96% alcohol (1 h), 100% alcohol (3 x 20 min), 50% (v/v) alcohol/chloroform (1 h) and finally chloroform (2 x 1 h). The dehydrated blocks were immersed in a wax bath containing a mixture of Sasolwax 5 kg) and beeswax (125 g) for 2 x 2 h. The blocks were finally imbedded in Sasolwax.

Sections (3 μ m) were cut onto glass slides with a Reichert Jung sliding microtome by Mrs Welthagen (Department of Pathology, Faculty of Veterinary Science, University of Pretoria). The sections were dried (70 $^{\circ}$ C; 1 h) and could be stored in this state for many months.

Prior to immune staining, the sections were dewaxed with xylol (5 min) and rehydrated with 100% alcohol (3 min), 96% alcohol (3 min), 70% alcohol (3 min) and finally H_2O (3 min). The sections were treated with 1% (v/v) H_2O_2 /methanol to inhibit endogenous peroxidase activity before being blocked and processed as described in 2.10.5. Once the sections had been dewaxed, they had to be processed within 24 h.

2.10.3 Preparation of cell lines for staining

Cell lines 15.4, 21.3 and normal type II sheep cells were grown in DMEM supplemented with 10% foetal calf serum (FCS). BRV and CFTH cells were grown in EAGLES medium with 10% bovine serum. For

immunocytochemical staining the cells were cultivated in 2-chamber slides (Miles Laboratories) for seven days, unless otherwise stated.

2.10.4 Reagents

Washing buffer: Tween 20 (Bio-Rad Laboratories) (0,5 ml) was added to 500 ml PBS to make a 0,1% Tween 20/PBS solution.

Blocking buffer: A 10% horse serum/PBS solution was used.

Primary antibodies: Monoclonal antibodies 2E3, 4A10, 11G11 and 59E11 were used at 1:100 dilutions. All sera were diluted with 10% horse serum/PBS. Sheep anti-MVV and goat anti-MPMV (p27) sera were used at 1:50 and 1:200 dilutions, respectively.

Affinity purified horseradish peroxidase-conjugated antibodies against primary antibodies (Peroxidase conjugates): Goat anti-mouse (H+L) and Rabbit anti-sheep IgG(H+L) peroxidase conjugates were from Dakopatts, Copenhagen, Denmark, and were both used at 1:200 dilution.

Peroxidase substrate: The substrate was made up freshly by dissolving diaminobenzidine hydrochloride (Sigma Chemical Co.) (10 mg) in 20 ml PBS. Immediately before use, 6 ul H₂O₂ (30% strength) was added.

2.10.5 Method

Frozen lung sections and cell slides were incubated in 70% (v/v)

ethanol/H₂O containing 1% H₂O₂ for 10 min (this procedure inactivates endogenous peroxidases) and air dried. The slides were incubated in blocking buffer for 30 min at room temperature. Monoclonal or polyclonal antibodies were added onto the slides, which were then incubated for 1 h in a humidity chamber at room temperature. Washing of the slides was done by pouring washing buffer over them, shaking them dry and repeating the procedure 3 times. The relevant peroxidase-conjugated second antibody was added to the slides and incubated for 1 h as before. After washing again as described, slides were rinsed with pure PBS and the substrate added. After 5 min the slides were washed under tap water and stained with haematoxylin for 1 min, then washed again under tap water. They were then air dried and transferred firstly to a glass slide holder containing 70% ethanol, then into xylol and mounted using Permount.

2.11 CONSTRUCTION OF JSRV GENOMIC LIBRARY

2.11.1 Methods

It was decided to make a library of the DNA extracted from a jaagsiekte lesion in a system that could express the proteins coded for by the inserted DNA sequences. It was hoped that with the use of the monoclonal antibodies and MPMV antiserum, the sequence coding for the relevant protein could be isolated and used as a possible jaagsiekte probe. For this application, cloning into the lambda phage gtl1 was chosen as it is a very efficient protein expression vector (Huynh, Young and Davis, 1985).

2.11.2 Purification of JS lesion DNA

DNA extracted by means of phenol from a JS lung lesion was mixed with an equal amount of caesium chloride (Boehringer Mannheim GmbH, West Germany) and 2 ml ethidium bromide (Sigma Chemical Co.) (10 mg/ml) and centrifuged (46 000 rpm; 48 h ; 15 C) in a Beckman Type 65 rotor. With the aid of UV light the DNA band was identified and collected by piercing the tube below the band. The DNA band was extracted twice with isoamyl alcohol and dialysed against three changes of TE buffer (pH 7,5) at 4 C. The DNA was recovered by precipitation with 2,5 volumes of absolute alcohol and 0,3 M sodium acetate (pH 5,2). After 2 h at -20 C the DNA was pelleted (10 000 rpm; 10 min) using a Beckman JA-20 rotor and the pellet resuspended in H₂O.

2

2.11.3 Preparation of 5 Kb fragments of JS lesion DNA

One ug JS DNA was digested with a dilution series of Eco RI enzyme, to determine the optimum concentration of enzyme required to obtain maximum yields of 5 Kb sequences. From this test digestion it was decided that 2 units of enzyme per 1 ug DNA was the optimum ratio. A large scale digest was then done on 158 ug JS DNA. The digested DNA was layered onto a 10 - 40% sucrose gradient and centrifuged (85 000 g; 24 h; 20 C) in a Beckman SW 27 rotor. The gradients were tapped and 1 ml fractions collected. Samples of the fractions were run on a 1% agarose gel to identify those fractions containing most of the 5 Kb segments. The fractions were combined and dialysed against 3 changes of TE buffer.

The DNA was extracted with butanol and precipitated with ethanol as described in 2.11.2.

2.11.4 Ligation and Packaging

A number of test ligations were done before the bulk ligation, to determine the optimal conditions for the reaction. The bulk ligation reaction mix consisted of: λ gt11 1 μ l (0,5 μ g); JS DNA 3 μ l (0,42 μ g); 10 X ligase buffer (300 mM Tris.HCl (pH 7,8), 100 mM MgCl₂, 100 mM DTT and 4 mM ATP); T4 ligase (1 μ l of 1,5 mg/ml) and H₂O (3 μ l), added together and incubated overnight at 16 °C. A sample was run on a 0,8% agarose gel alongside pure λ gt11 and it was shown that the JS/gt11 DNA ran slower than the pure gt11, thus indicating that the ligation had worked. The ligated JS DNA/gt11 DNA was then packaged into DNA using in vitro packaging kits (Amersham International plc.) and amplified on E. coli Y1090 (recA-). A total of 12 packaging kits were used which, after amplification, gave 500 ml of recombinant phages containing $3,9 \times 10^6$ plaque forming units (pfu) per ml, viz. a total $1,95 \times 10^9$ pfu.

2.12 SCREENING OF λ GT11 DNA LIBRARIES WITH ANTIBODY PROBES

Phage λ gt11 forms plaques on a lawn of E. coli Y1090. These plaques can be easily screened to identify those plaques that contain genetic material that codes for a specific protein, using an immune overlay technique. This is done by overlaying the plaques with nitrocellulose which binds proteins non-specifically. After blocking the vacant sites on the nitrocellulose, high titred

antibodies are layered onto the nitrocellulose and a method similar to the immunoblot technique, is used. The method used to screen the libraries is an adaption of that described by Huynh et al., 1985.

2.12.1 Reagents

LB broth: Bacto-tryptone (10 g), yeast extract (5 g) and NaCl (10 g) were dissolved in 1 litre distilled water. The mixture was autoclaved for 15 min, cooled to about 45 C and aliquoted into 100 ml bottles.

Top agarose: Agarose (Miles Laboratories (pty) ltd.) (0,7 g) was dissolved in 100 ml LB broth and autoclaved.

Agar plates: Agar (Sigma Chemical Co.) (15 g) was dissolved in 1 litre LB broth and autoclaved. After cooling to 45 C, Ampicillin (Sigma Chemical Co.) (10 mg) was added. The mixture was poured into sterile petri dishes (15 ml/plate) and stored at 4 C for up to 1 month.

Isopropyl B D-thiogalactopyranoside (IPTG): A 10 mM IPTG (Sigma Chemical Co.)/H₂O solution was made from a 1 M stock solution stored at -20 C.

2.12.2 Method

A scraping of E. coli Y1090 was seeded into 100 ml L broth, containing 0,001 g Ampicillin. This was shaken overnight at 37 C. The following day 50 ul of a 1:10 (2×10^4 pfu) dilution of the JS

lesion DNA library was added to 200 ul of an E. coli Y1090 overnight culture. After 20 min incubation at 37 C, 50 ul X-gal was added to each tube, to which 4 ml top agarose was added. Working quickly, the top agarose mixture was added to agar plates that had been pre-incubated at 42 C. The plates were then incubated at 42 C for 3 h. Fifteen minutes before the incubation time was up, a nitrocellulose circle (Amersham International plc.) for each agar plate was soaked in 10 mM IPTG and dried on filter paper. The moist nitrocellulose circles were carefully layered onto the incubated plates and incubated for a further 3 h at 37 C. The plates and nitrocellulose were marked so that the nitrocellulose could be replaced in exactly the same position so as to locate the positive plaques at a later stage. The circles were removed and transferred to 50% horse serum/PBS and incubated for a further 1 h. The nitrocellulose was then overlayed with a mixture of monoclonal antibodies viz 2E3, 4A10 and 11G11 and processed as described for the immunoblot technique (2.8.2.2).

2.13 ISOLATION OF JSRV RNA

2.13.1 Virus purification

Freon-treated JS lung wash pellets (2 ml) were layered onto 6 sucrose gradients (20 - 50%) and centrifuged overnight using a Beckman SW 27 rotor (85 000 x g; 16 h; 4 C). Fractions (2 ml) were collected and their densities determined. Fractions either side of the 1,186 g/ml fraction were combined and used directly as the source of JSRV RNA.

2.13.2 JSRV RNA isolation

A modification of the method described by Chirgwin, Przybyla, MacDonald and Rutter, (1979) was used to isolate RNA from JSRV-containing sucrose gradient fractions. This method was designed to minimize breakdown of RNA by contaminating RNases. All centrifugations were done in a Beckman JA-20 rotor, unless otherwise stated.

Gradient fractions either side of and including the 1,186 g/ml density fraction were combined in a 30 ml Corex tube. For every 10 ml gradient fractions, the following were added. Guanidinium hydrochloride (Merck, Schuchardt and Co.) (21 g); 2-Mercaptoethanol (2,1 ml); 1 M sodium citrate buffer (pH 7,0) (0,75 ml) and 10% sarcosyl (1,5 ml). The mixture was heated for approximately 1 min at 60 °C to dissolve the guanidinium hydrochloride. Finally 1 M acetic acid (0,5 ml) was added. The mixture was centrifuged (10 000 rpm; 10 min; 25 °C) to remove any insoluble particles. The mixture was then divided into two 30 ml Corex tubes (15 ml/tube) to which 11 ml absolute alcohol was added. The tubes were incubated overnight at -20 °C and the following day the RNA was pelleted (15 000 rpm; 45 min; 0 °C). The pellet was dissolved in 3,6 ml RNase free H₂O; 0,2 ml 2-mercaptoethanol and 0,4 ml 3 M sodium acetate buffer (pH 5,2). If necessary the mixture was then clarified (10 000 rpm; 45 min; 0 °C). The RNA was precipitated for a second time by adding 10 ml absolute alcohol (0 °C) to the mixture and incubated for a minimum of 2,5 h at -20 °C. The RNA was recovered by centrifugation (15 000 rpm; 45 min; 0 °C). The

pellet was freeze-dried, dissolved with the minimum volume of RNase-free H₂O and stored at -20 °C.

2.14 PURIFICATION OF POLY-A RNA

The RNA of retroviruses is polyadenylated at the 3' end. It is therefore possible to further purify the viral RNA from other contaminating non-polyadenylated RNA's. At the time of this research a new affinity paper for this purpose was marketed by Amersham International plc. This product proved to work very well and was used in the purification of JSRV mRNA. The procedure is very simple and quite efficient.

2.14.1 Reagents

All reagents were from Merck, Schuchardt and Co., unless otherwise stated. Deionized, distilled water and glassware were treated with 0,1% diethylpyrocarbonate (DEPC) (Merck, Schuchardt and Co.) to remove RNAase activity as described in Maniatis, Fritsch and Sambrook (1982). Great care was taken and gloves were used when handling all buffers, which were autoclaved before use, to prevent RNase contamination.

Buffers: A 2 x SSPE buffer was made by dissolving NaCl (1,74 g), NaH₂PO₄ · H₂O (0,312 g) and EDTA (disodium salt) (0,074 g) in 100 ml H₂O.

2.14.2 Method

Gloves were worn at all times. A square piece (0,5 cm²) of Hybond-

mAP paper (Amersham International plc.) was cut off the stock sheet. This piece was saturated with 2 x SSPE buffer and placed onto a sheet of 3 mm blotting paper (Whatman) or if the RNA was in a large volume, the Hybond-mAP paper was placed on parafilm to slow the passage of liquid so as to maximize the binding. The RNA was pipetted onto the mAP paper in small volumes and allowed to dry between each application. The mAP paper was washed for 10 min in 0,5 M NaCl (2,5 ml). This was repeated twice more. After the third wash the mAP paper was placed in 70% (v/v) ethanol/H₂O for 2 min, then blotted on 3 mm Whatman paper. The paper was transferred to an Eppendorf tube to which 75 ul H₂O was added (just enough to cover the paper) and incubated in a 70 °C water bath for 5 min. The mAP paper was transferred, using forceps, to a second Eppendorf tube and the elution procedure repeated. The poly-A RNA remained in the water.

The results obtained when Hybond-mAP was used to purify JS mRNA are tabulated in Table I.

TABLE I: Purification of JSRV RNA using Hybond-mAP affinity paper.

	Volume	Total RNA	% RNA recovered
Before mAP	25 ul	50 ug	
Elution No 1	55 ul	5,5 ug	11 %
Elution No 2	85 ul	4,3 ug	9 %

2.15 SYNTHESIS OF JSRV CDNA

2.15.1 Introduction

The ability to synthesize complementary DNA (cDNA) copies of mRNA (and their subsequent cloning) has proved to be an immensely powerful and versatile technique in molecular biological research. In the JS system, this would mean that the DNA copy of JSRV RNA could be inserted into a bacterial or other system in which the genetic material can be amplified and the the proteins coded for could possibly be expressed in large amounts. In view of the shortage of JSRV and the inability to cultivate the virus in vitro, this would be a breakthrough for JS research.

In short, the technique of cDNA synthesis and cloning involves, firstly, the synthesis of a double stranded DNA copy of the RNA of interest. The cDNA is then inserted into bacterial or viral based vectors and the cDNA recombinant introduced into prokaryotic or eukaryotic cells where replication takes place. This results in amplification of the cDNA sequences and, if suitable control sequences have been introduced, synthesis of the protein coded for by the original mRNA.

2.15.2 Methods

To synthesize the double stranded cDNA of JSRV RNA an Amersham cDNA synthesis kit was used. To report on the reagents and method used in this thesis, would merely duplicate a well-researched protocol that comes with the kit. The points to note were that

0,65 ug JSRV RNA, instead of 1 ug, was used in the 1 ug reaction as described in the protocol. A total of 0,033 ug double stranded cDNA was synthesized. A table reporting the percentage yield obtained for the first and second strand synthesis is presented below.

TABLE II: The synthesis of first and second strand cDNA and percentage yields obtained using the Amersham cDNA synthesis kit and JSRV mRNA.

	Starting concentration	cDNA	% Transcription
1 st strand	0,65 ug	0,0365 ug	5,6 %
2 nd strand	0,0365 ug	0,0328 ug	89,7 %

The yields obtained for JSRV cDNA are close to the yields obtained when 1 ug control globin mRNA was used. The yields were as follows:

% mRNA transcribed for the first strand cDNA: 15 - 30%

% first strand cDNA transcribed into second strand cDNA: > 90%.

With these figures in mind a yield, for JS, of 5,6% for the first strand synthesis is possibly a little low, however, 89,7% for the second strand is acceptable. The synthesized cDNA was used in cloning experiments performed by a colleague, as it formed part of her project, hence the methods used are not reported here.

2.16 SCREENING OF CLONES CONTAINING JSRV cDNA INSERTS

2.16.1 Introduction

JSRV cDNA was inserted into pUC 13, which is a good expression vector when it is transformed into E. coli JM105. The recombinant genes in the transformed E. coli are transcribed into mRNA which in turn are translated into proteins. The proteins are produced inside the E. coli which have to be lysed to release them for further analysis. To achieve this the transformed E. coli, containing the recombinant plasmids, were grown on nitrocellulose circles which had been placed on top of prepared agar plates. The agar contained the required nutrients for E. coli growth. The E. coli formed fairly large colonies overnight and were ready to be screened the following day. The cultures were screened with MAb's 2E3, 4A10 and 11G11, to identify those colonies expressing a related antigen. Two methods were used to lyse and screen the colonies, the salt lysis method giving the best results.

2.16.2 Chloroform method

This method is a slightly modified version of that described by Helfman (1983). The nitrocellulose containing the transformed E. coli colonies were exposed to chloroform vapour for 15 min to lyse the colonies. The nitrocellulose was then incubated overnight at room temperature in 10 ml 50 mM Tris-HCl buffer (pH 7,5), containing 150 mM NaCl, 5 mM MgCl₂ and 3% Bovine serum albumin to which DNase (1 ug/ml) and lysozyme (40 ug/ml) were added. After washing in a 50 mM Tris-HCl buffer (pH 7,5), containing 150 mM

NaCl, the nitrocellulose circles were incubated in the primary antibody for 2 h with constant rocking. The following steps viz. washing, peroxidase conjugate and substrate were exactly as described in 2.8.2 for the immunoblot.

2.16.3 Salt lysis method

This was the preferred method as it was a lot quicker and cheaper. The protocol followed was that described by Puhler and Timmis (1983). The nitrocellulose circles containing the transformed E. coli colonies that had been growing overnight were placed onto Whatman 3 mm filter paper discs, saturated with 0,5 M NaOH, for 5 min. They were then transferred to filter paper discs saturated with 1 M Tris-HCl buffer (pH 7,0), for 2 min to neutralize the NaOH. This step was repeated. The circles were transferred to filter paper discs saturated with 1 M Tris-HCl (pH 7,0) containing 1,5 M NaCl, for 5 min. Finally, they were transferred to filter paper discs saturated with 20 mM Tris, 500 mM NaCl (pH 7,5) for 4 min. This last step was repeated. After washing in PBS (3 x 5 min) and blocking in 50% horse serum/PBS (30 min), they were incubated in the relevant primary antibody. Following this step, the procedure was the same as described in 2.8.2 for the immunoblot.

2.17 NEGATIVE STAIN ELECTRON MICROSCOPY

Viral samples were negatively stained in preparation for observation with a transmission electron microscope using the method described by Payne and co-workers (1983). Briefly, Formvar carbon-

coated grids were floated consecutively on drops of virus suspensions, 2,5% gluteraldehyde in 0,1 M cacodylate buffer with 4% sucrose (pH 7,2) (GA fixative), distilled water and either 3% phosphotungstic acid (PTA) (pH 6,0) or 2% uranyl acetate (pH 4). The time on each droplet varied between 10 and 60 seconds.

CHAPTER 3

THE JAAGSIEKTE RETROVIRUS

3.1 INTRODUCTION

Research on the jaagsiekte disease complex has been beset with many problems, the most important one being the inability to cultivate the etiological agent in vitro (Verwoerd et al., 1983). In this chapter the etiological agent is referred to as the jaagsiekte retrovirus (JSRV). Mention is made of this fact because certain findings, discussed later in this thesis, suggest that there could be other agents also involved in the pathogenesis of JS.

As JSRV cannot be cultivated in vitro yet, research was reliant upon experimentally infected lambs as the only source of virus. Virus recovered from the lung washes is in relatively low concentrations and associated with and attached to non-viral lung debris (personal observation). For these reasons, direct monitoring of the virus by TEM was almost impossible. Fortunately with the discovery that a retrovirus was involved (Perk et al., 1974 and Verwoerd et al., 1980), it was possible to detect the virus using an RNA dependant DNA polymerase (RDP) assay. One shortfall of this assay is that it is partially inhibited in the presence of lung debris (York, 1984), another is a lack of specificity as it would also detect other retroviruses present in the sheep lungs.

Preliminary research on JSRV was concerned with the purification of the virus from the lungs of infected sheep (York, 1984). These purifications were monitored with the RDP assay as well as SDS-PAGE which was used to demonstrate those proteins that were consistently associated with high RDP activity. Although the purification procedures developed provided sufficient purification to identify the viral proteins, even the best preparations were still shown to contain significant amounts of immunoglobulins and other non-viral material. This meant that sera produced against these viral preparations, even after extensive absorption, were still not specific enough for diagnostic or research purposes (Verwoerd et al., 1983).

With the advent of MAb technology, it became possible to produce JS-specific MAbs by using partially purified JSRV as the antigen. The preparation and application of such JS-specific MAbs and their uses and limitations in the immunological characterization of JSRV form a major part of this chapter.

A major limitation of the JS specific MAbs studied was their inability to react in an immunoblot technique. This is a common problem encountered with MAbs. The problem was circumvented by using a polyclonal serum against the group-specific antigen of Mason-Pfizer monkey virus (MPMV) (p27) which cross-reacts with a similar sized protein in JS lung wash material (Sharp and Herring, 1983). This polyclonal serum was used in an immunoblot technique to identify JSRV and also to distinguish it from Maedi Visna virus

(MVV). This serum and technique was also used to show the serological relationship between JSRV and a newly isolated bovine retrovirus (BRV).

This chapter therefore deals with the purification and characterization of the JSRV, with the emphasis on the development of immunological reagents and techniques for comparing it with and differentiating it from the other two viruses described in chapters 4 and 5.

3.2 RESULTS

3.2.1 Biochemical characterization of JSRV

3.2.1.1 JSRV purification

A large part of the preliminary JS research was aimed at the development of a purification procedure for the JSRV. Since most of that work has previously been presented in the author's M.Sc. thesis, it will not be discussed in detail again. However, a few purification procedures and results will be presented because they are particularly relevant to this study.

The purification procedure that gave the best results, when monitored using RDP specific activity as a measure of purity, is a combination of organic solvent extraction, rate zonal centrifugation and permeation chromatography. The results obtained when this combination was used are presented in Table 1.

TABLE 1 Purification of JSRV from lung rinse pellets, monitored at each stage for RDP activity and protein content.

Fraction	see method	Total RDP-activity cpm	Total Protein mg	lPurification factor
Lung rinse pellet	2.3.1	$4,7 \times 10^6$	21,2	1,00
After Freon	2.4	$6,34 \times 10^6$	12,6	2,27
Percoll band	2.5.1.3	$4,7 \times 10^6$	7,8	2,70
Viral peak from Sephacryl S1 000	2.6	$7,61 \times 10^6$	0,12	286,00

lPurification factor = $\frac{\text{Total RDP activity}}{\text{Total protein content}}$, normalized to 1 for starting material.

Samples were taken from the different stages of the purification and analysed for purity by SDS - PAGE and also to calculate the size of the likely JSRV polypeptides (see Plate 1).

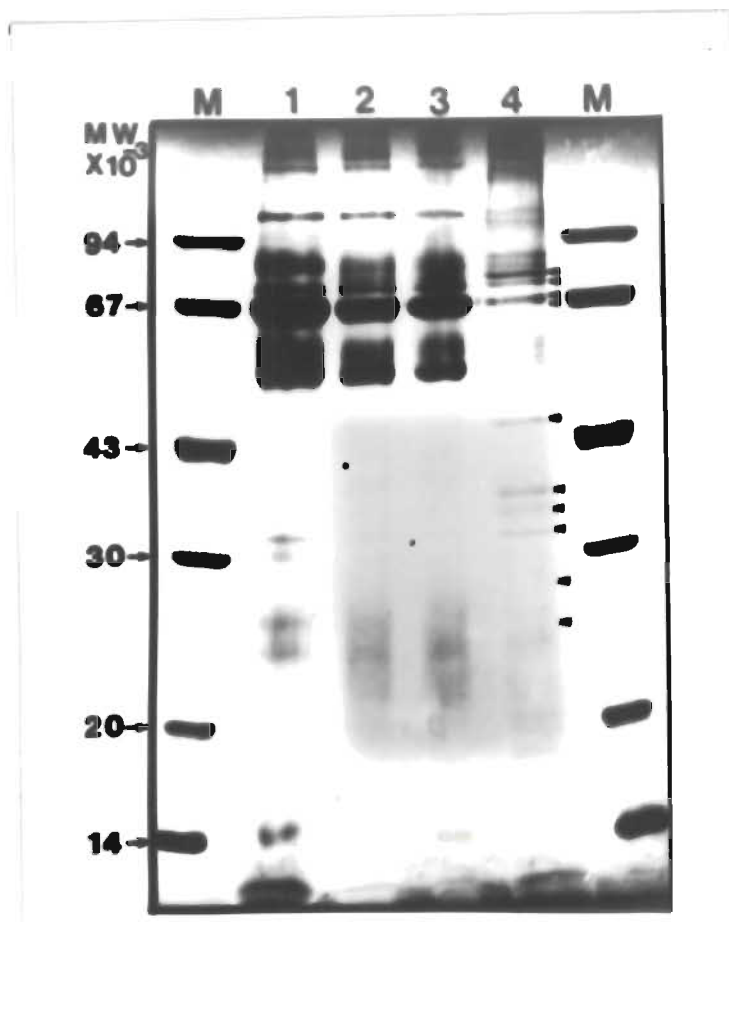
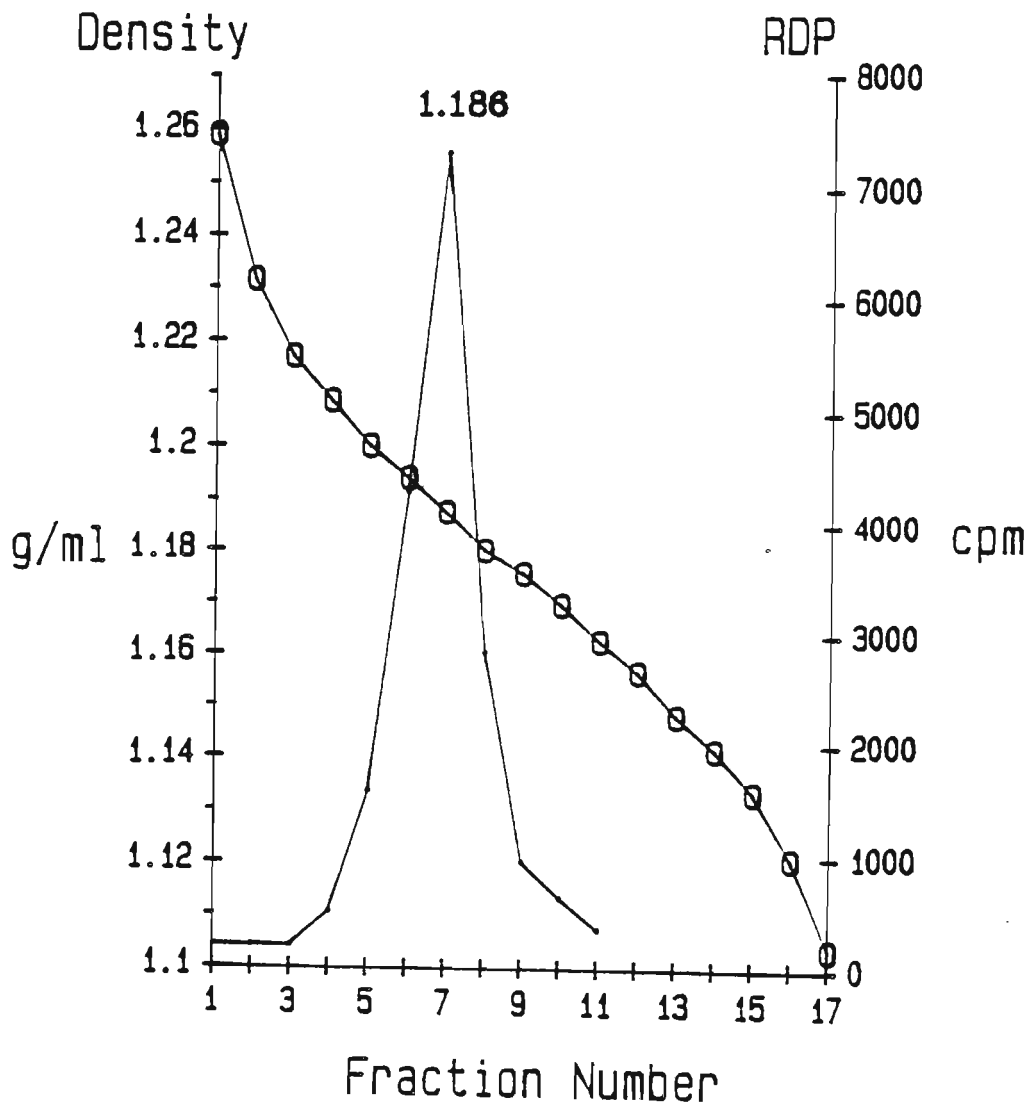


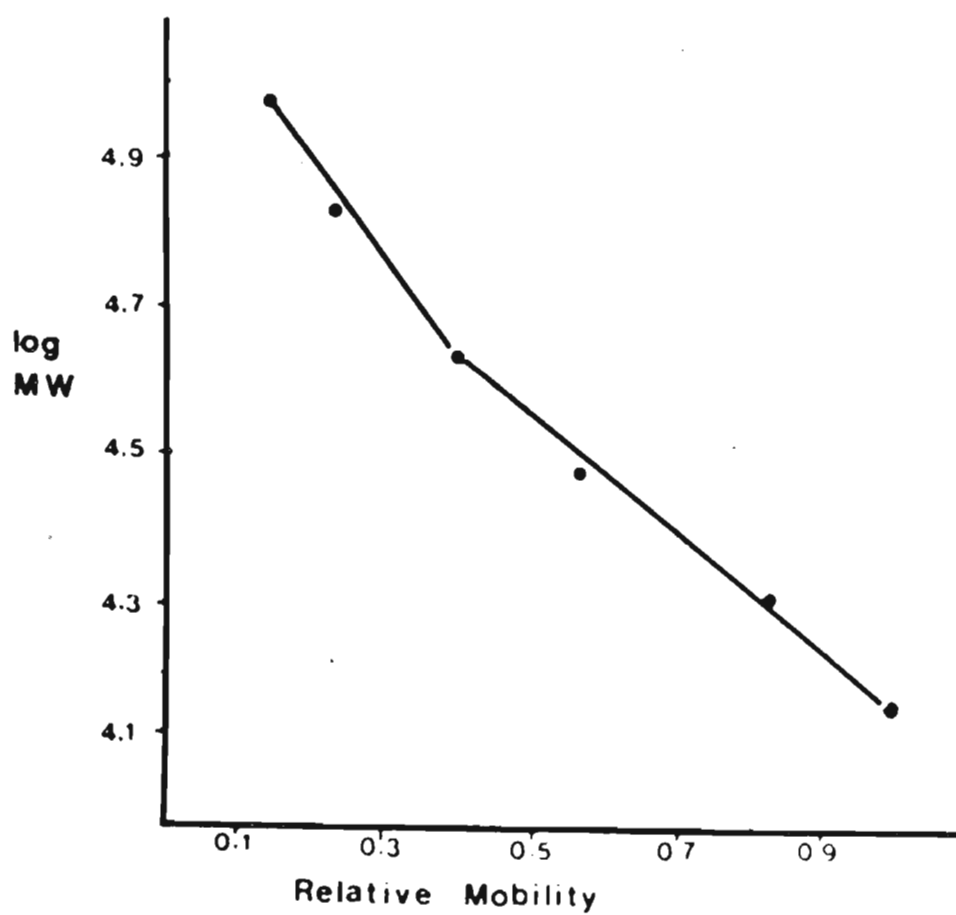
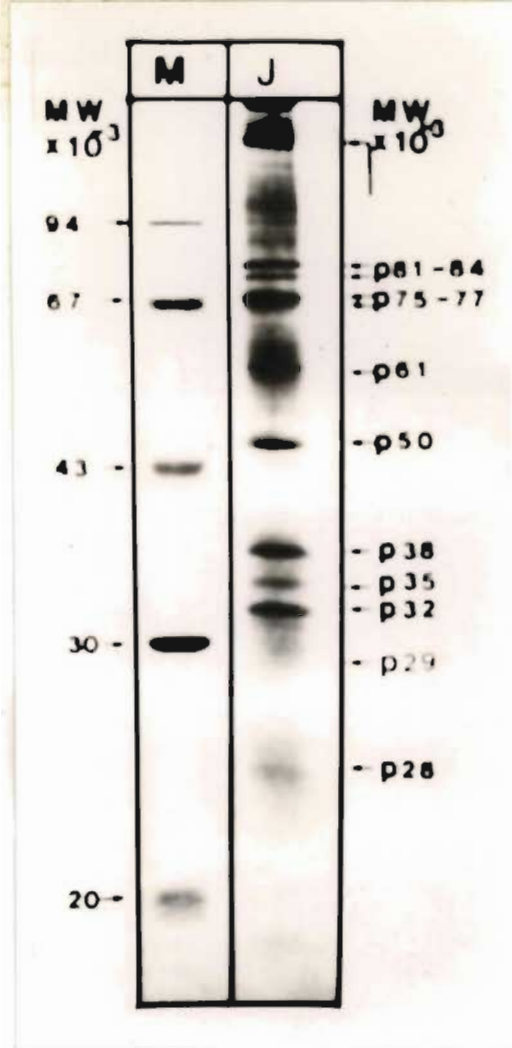
Plate 1 SDS-PAGE of samples taken at different stages of the JSRV purification. Electrophoresis was in a 10% polyacrylamide gel run for 12 h at 9,5 v/cm (see 2.8.1.2). Lane 1, represents JS lung rinse pellet. Lane 2, same as lane 1 after Freon treatment. Lane 3, as in lane 2 but after Percoll gradient centrifugation. Lane 4, as in lane 3 but after gel chromatography. Lane M, represents low molecular weight markers (Pharmacia, Uppsala, Sweden). (), indicates considered JSRV polypeptides.

Another purification procedure that is frequently used in retrovirus purifications is isopycnic centrifugation using sucrose or caesium chloride as the gradient medium. Although the purity achieved was not as good as with the Freon, Percoll and column procedure, isopycnic centrifugation allowed the calculation of the bouyant density of the virus. As density is used as a characteristic in classifying retroviruses, it was important to determine the density of JSRV. From the results presented in Fig. 1 the buoyant density of JSRV was estimated to be 1,186 g/ml in sucrose at 5 C.



3.2.1.2 Polypeptides of JSRV

A low molecular weight marker calibration kit from Pharmacia, Uppsala, Sweden, was used to estimate the molecular weights of the JSRV polypeptides. The calibration curve (Plate 2b) was constructed from the polypeptide pattern shown in Plate 2a. The currently known JSRV polypeptide pattern consists of nine proteins designated p26; p29; p32; p38; p50; p75; p77; p81; and p84. Additional evidence has been presented in the author's MSc thesis that supports the contention that p26; p29; p32; p81 and p84 as being likely JSRV polypeptides, whereas, p50 appears to be a protein shared by normal lung tissue and JSRV. More evidence in support of this was obtained from studies with one of the JS MAbs.



3.2.2 Immunological characterization of JSRV

3.2.2.1 Isolation of JS-specific MAbs

The advent of MAb technology allowed researchers for the first time to produce specific antibodies using impure sources of antigen. In the JS system, where even the most highly purified preparations are still contaminated with non-viral lung material it was hoped that MAbs would be the breakthrough required to obtain JS-specific antibodies.

An initial fusion between Sp2 myeloma and spleen cells from a mouse immunized with purified JS material, was partially successful. Although the fusion itself worked well and 70% of the wells were positive for JSRV antigen, screening against normal lung antigens eventually showed that all the clones secreted antibodies that reacted with normal lung (NL) proteins, mostly IgG and IgA. For this reason, a second fusion was performed but this time all the clones were screened, in a duplicate ELISA, against both JS and NL antigens. In this way those clones producing antibodies against the major normal lung antigens were eliminated.

This approach worked well in the subsequent fusion and 10 clones were isolated that produced antibodies with much higher titres against JS than against NL material. Further screening resulted in the selection of three of the clones, which were named 2E3, 4A10 and 11G11.

A fourth clone, namely 59E11, was also included in this study

because it was shown to be specific for IgA, a major contaminant in JS infected lungs. Clone 59E11 was isolated from the first fusion.

Had JSRV been produced in vitro, it would have been possible to label the viral proteins using radioactively labelled amino acids and immune-precipitate the protein(s) against which the MAbs were directed. The immune precipitation technique requires either a large amount of antigen or radioactively labelled proteins to increase the sensitivity. In the case of JSRV neither prerequisites were fulfilled and a number of alternate experiments had to be performed to determine whether the MAbs were JS specific.

3.2.2.2 Determination of the immunoglobulin subclass of the monoclonal antibodies

A monoAb-ID EIA kit from Zymed Laboratories Inc. was used to determine the subclasses of the different MAbs (see Methods 2.9.4). Table 2 shows the subclasses and light chain type for the four MAbs.

TABLE 2 Subclass and light chain component of four MAbs.

MAb	Subclass	Light chain
2E3	IgG1	kappa
4A10	IgG3	kappa
11G11	IgG1	kappa
59E11	IgG2a	kappa

3.2.2.3 Specificity of the MAbs

To confirm that MAbs 2E3, 4A10 and 11G11 were specific for JS material a series of JS and NL wash pellets were diluted (1:400) with carbonate buffer, bound to microtitre plates and tested for the presence of antigens with which the MAbs reacted (see Methods 2.2.2). The results are presented in Table 3.

TABLE 3 ELISA result showing the specificity of MAbs 2E3, 4A10 and 11G11 when JS and NL wash pellets were diluted 1:400 and used as antigen.

Sheep tested	No	MAbs					
		2E3	%+VE	4A10	%+VE	11G11	%+VE
JS +VE lung	17	15	88	15	88	17	100
Normal lung	76	23	30	3	4	15	20

The results presented in Table 3 indicated that the MAbs were detecting antigen in a low percentage of the normal lungs as well. To exclude the possibility that they might be against other non-viral lung antigens which commonly occur in sheep lungs, a selection of possible contaminants were also used as antigen in an ELISA. These results are presented in Table 4.

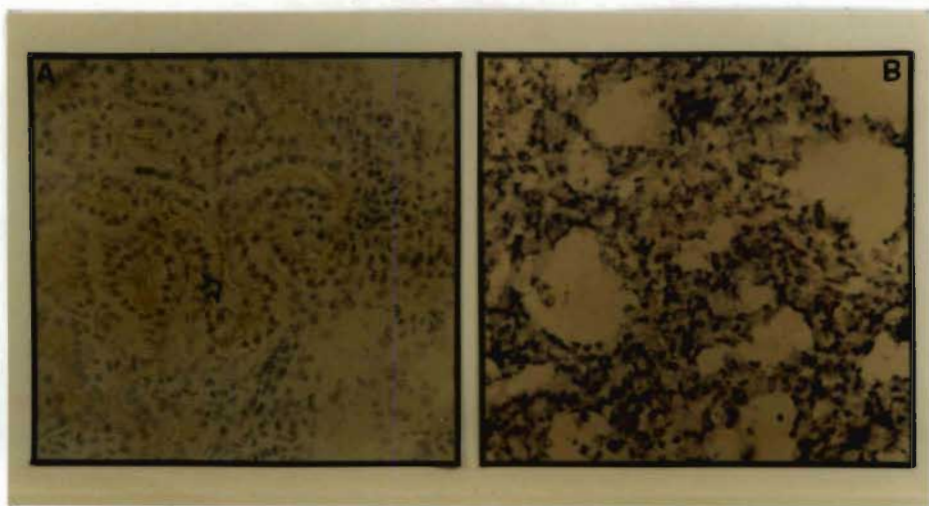
TABLE 4 The results obtained when MAbs 2E3, 4A10 and 11G11 were screened against some common contaminants present in sheep lungs using an ELISA.

Antigen	2E3	4A10	11G11
IgA	- VE	- VE	- VE
IgG	- VE	- VE	- VE
Surfactant	- VE	- VE	- VE
<u>Mycoplasma arginini</u>	- VE	- VE	- VE
<u>Mycoplasma ovipneumonia</u>	- VE	- VE	- VE
<u>Lentivirus</u>	- VE	- VE	- VE
Macrophage	- VE	- VE	- VE

The three MAbs were also tested against frozen sections prepared from JS infected and normal lung sections using an indirect immunoperoxidase technique (see Methods 2.10). A summary of these results is presented in Table 5.

TABLE 5 A summary of the observations made when MAbs 2E3, 4A10 and 11G11 were used to detect antigen in frozen JS and normal lung sections using an indirect immunoperoxidase technique (see 2.10).

		MAbs								
		2E3			4A10			11G11		
		No	+VE	%	No	+VE	%	No	+VE	%
Frozen lung section tested										
JS +VE	13	13	100		7	7	100	16	16	100
Normal lung	5	2	40		3	0	0	5	3	60



There was an unexpectedly high percentage staining of normal lungs with MAb 11G11. However, there was always much more intense staining in JS than in the normal lungs. In addition, the staining in JS sections was at the tumour cell surface and in the lumen (see Plates 5a and b), whereas it was localized to the bronchioles in the normal lung sections. The significance of this is not clear at this stage.

MAb 2E3 also stained a significant number of normal lungs. Here, as with 11G11, staining was localized to the bronchioles in normal lung sections. Staining in JS sections was also at the tumour cell surface and in the lumen. However, there were tumour lesions that did not stain with 2E3 (see Plates 3a and b). This was in agreement with the EM observations which showed that not all tumour lesions produced JSRV (Payne, 1985).

Staining with 4A10 was more specific than with either 2E3 and 11G11. Although there were transformed areas that did not stain with 4A10, most of the staining was at the tumour cell membrane. The normal lung sections did not stain at all (see Plates 4a and b).

MAb 59E11, which is against IgA, stained the JS sections very intensely and extensively. In contrast to this, there was very little staining in the normal lung sections, suggesting that there is a much higher concentration of IgA in JS infected lungs than in normal lungs. This is in agreement with earlier theories (Verwoerd et al., 1983).

The MAbs were also used to get an overall picture of the distribution of the antigen which they detect in frozen sections of a JS infected lung. Three areas can be distinguished in JS lungs viz. 1) the normal uninfected areas, 2) the areas where transformation has just been initiated and 3) the consolidated areas - the most advanced stage of tumour formation. Frozen sections were prepared from these three areas (see 2.10.1) and stained using MAbs 2E3, 4A10 and 11G11. Table 6 presents a summarized result of the distribution of the MAb related antigens in a JS lung.

TABLE 6: Distribution of MAb related antigen(s) in a JS infected lung. Results were obtained using an indirect immunoperoxidase technique on frozen sections taken from three regions of a JS infected lung (see 2.10.1).

Area of JS infected lung	MAbs		
	2E3	4A10	11G11
Normal	mostly -ve	-ve	mostly -ve
Early lesion	+ve	+ve	+ve
Consolidated	-ve	-ve	-ve

It is important to note the absence of staining in the consolidated areas of JS infected lungs, with MAbs 2E3, 4A10 and 11G11. As previously observed, there was still some positive staining in the normal areas with MAb's 2E3 and 11G11. Once again the MAbs were very useful in monitoring the distribution of related antigen in an infected lung. It seems that the early lesion contains most of the virus and the consolidated areas, which are the sites of

advanced transformation, contain very little virus.

In addition to testing the MAbs against frozen lung sections it was also investigated whether the MAbs detected any related antigens when the sections were fixed in formalin and mounted in paraffin (see 2.10.2). Paraffin sections have an advantage over frozen sections in that cell definition is much clearer. Unfortunately, fixing the sections in formalin often has a detrimental effect on the antigenicity of the proteins.

Paraffin sections of normal lung and JS lesion taken from the same lung, were overlayed with the different MAbs. Interestingly, only MAb 4A10 reacted on these sections. In plate 6a, which shows the section of JS lesion area (the cauliflower-type arrangement), the surface of the tumour cells clearly stain brown. In the normal lung section (Plate 6b) no brown staining is observed (note the large air-filled areas).

Although all the tumour cells, seen in Plate 6a, show positive brown staining, there were many tumour cells that did not stain up. This might be what one would expect to see if the MAb was against a viral antigen, as it is believed that not all tumour cells produce virus.



Plate 6a A plate showing that MAb 4A10 reacts with antigen present on the surface of JS tumour cells (brown staining). The lung sections were fixed in formalin and embedded in paraffin (see 2.10.2)

b A plate showing that MAb 4A10 does not react with normal lung sections taken from the same lung and prepared as described in the legend to Plate 6a.

Having tested the MAbs against JS lung sections, it was decided to investigate whether the MAbs reacted with any antigen being produced in a cell culture that had been established from JS tumour cells.

In this laboratory Coetzee and co-workers (1976) established a cell line, designated 15.4, from a field case of JS. These cells were shown to transmit JS when injected intratracheally into newborn lambs. Since then other cell lines, namely 21.3 and 69.1, have been established, following the same procedures. Two of these JS tumour cell lines, namely 15.4 and 21.3, were used in this study. From infectivity trials and RDP-assays these cells were known to contain low levels of the JS etiological agent (Verwoerd, unpublished results). For this reason the MABs were used to investigate whether any related antigen was being produced in these cells. These cells were grown in 2-chamber slides (see 2.10.2) and MAB-related antigen was detected using an indirect immunoperoxidase technique (see 2.10.4). A normal type II cell culture was used as control. In Plates 7, 8 and 9, it is clearly evident that all three MABs detected related antigens in the tumour cell lines while the control cells were negative.

The production of the antigen detected by the MABs in tumour cell lines was thought to be influenced by the stage of cell growth. To investigate this both cell lines, viz. 15.4 and 21.3, were seeded onto culture slides (see 2.10.3) and stained with MAb 11G11 on different days after seeding. Table 7 shows the percentage of cells that stained positive per one thousand cells counted.

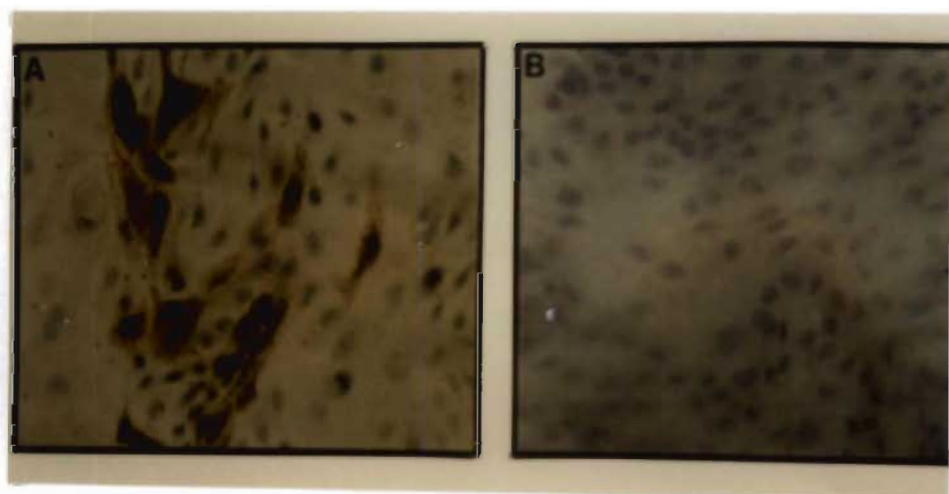
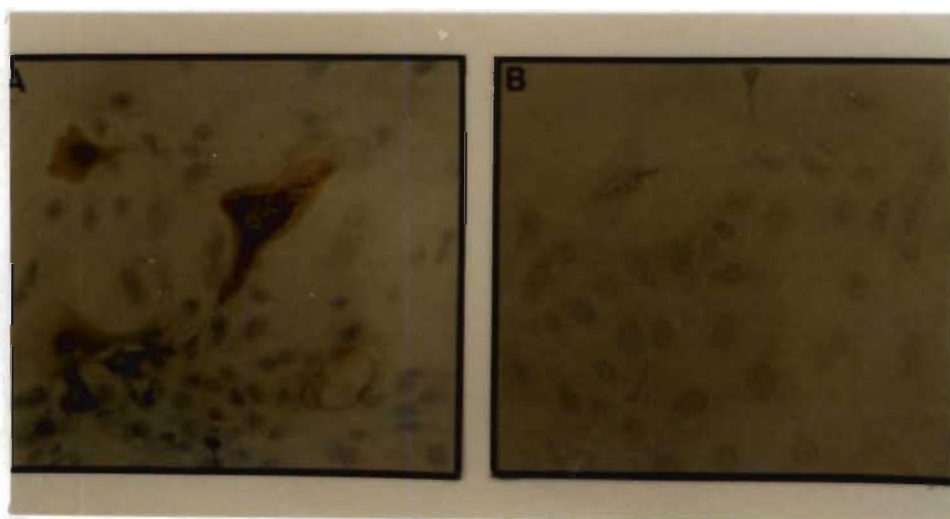
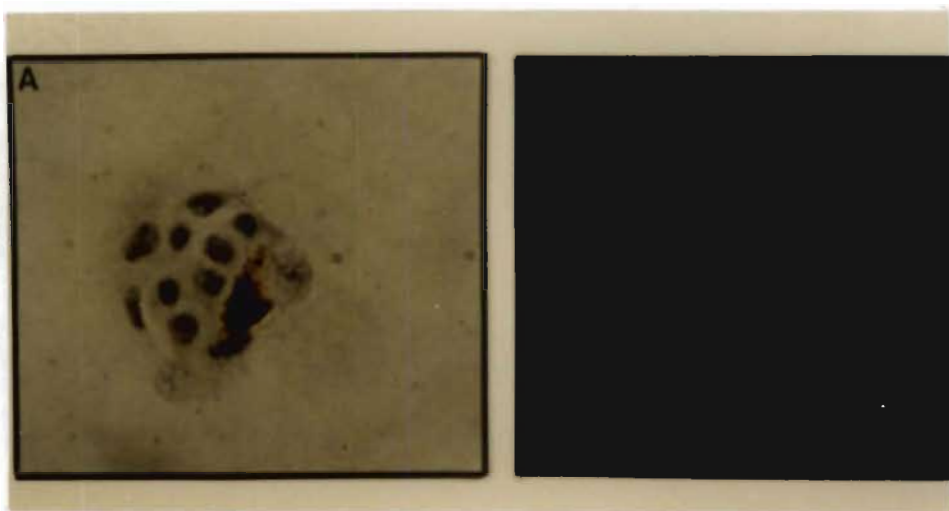


TABLE 7 The percentage of cells producing antigen detected with MAb 11G11, in 15.4 and 21.3 JS tumour cell lines. Production was monitored at different stages of cell growth using an indirect immunoperoxidase technique.

Cell line	Day 4	Day 7	Day 11	Day 14
15.4	1,0%	0,4%	2,8%	4,0%
21.3	0,8%	0,8%	1,3%	10,0%

As can be seen in Table 7, the percentage of cells that produce antigen varies from 0,4% to 10,0%, depending on the cell line and the stage of growth. Maximum production of antigen was seen after two weeks when the cells were near confluency. Normal type II cells which were included as the control, did not show any antigens that reacted with the MAbs. The percentage of cells that stained up was in agreement with predicted values which were based on RDP assay and infectivity results. It appears therefore, that the MAbs can be used to monitor virus production using an indirect immunocytochemical staining method.

The evidence presented thus far indicated that all three MAbs were reacting with antigens associated with jaagsiekte. It also appeared that MAbs 2E3 and 11G11 were reacting with tumour-related antigens and that MAb 4A10 was virus-specific. The high number of normal lung wash pellets that were positive with MAbs 2E3 and 11G11, using the ELISA, and the significant staining of normal lung sections suggested that these two MAbs were present in low levels in normal lung material. It therefore remained for the assay conditions to be optimized so that the MAbs could be used in

the development of diagnostic and JS-specific research tools. The application of the MAbs in this regard is discussed in the following section.

3.2.2.4 Application of the MAbs

3.2.2.4.1 Detection of antigen by means of an ELISA

In Table 3 JS and normal lung wash pellets were diluted 1:400 and 50 μ l bound to the microtitre plates. This approach can be very misleading as it does not take into account the effect of concentration. The size of the normal lungs differ greatly from those infected with JS, therefore a concentration effect might well influence the detection of antigen. In an attempt to investigate the possible use of MAbs in the development of a diagnostic test for JSRV, it was decided to standardize the assay. Firstly, to standardize the antigen concentration, protein determinations were done on a random selection of JS and normal lung wash pellets. The pellets were then diluted so that 1 μ g of protein was bound per microtitre well. Secondly, the MAbs were also used at dilutions that gave the highest JS positive:normal ratio. The results of this investigation are shown in table 6. They confirm the presence of antigens, with which the MAbs react, in JS lungs and that the MAbs could be used in the development of a diagnostic test.

TABLE 8 Results of an ELISA when JS and normal lung wash pellets were diluted so that 1 ug was bound to each well. MAb 4A10 and 11G11 were used at 1:100 and 1:500 dilutions, respectively, to detect related antigen.

	Sheep number														
	1	2	3	4	5	1	2	3	4	5	6	7	8	9	10
Ag	N	N	N	N	N	J	J	J	J	J	J	J	J	J	J
Ab															
4A10	0	0	0	0	0	1+	1+	1+	1+	1+	1+	2+	0	0	1+
11G11	0	0	0	0	0	3+	8+	6+	4+	6+	5+	9+	3+	2+	3+

0 = below background

+ = number of times greater than background

N = normal lung wash pellet (1 ug/well)

J = jaagsiekte lung wash pellet (1 ug/well)

It was encouraging to observe that the false positive reaction between the MAbs and normal lung wash pellets could be eliminated by using the correct antigen dilution. These conditions and techniques were used to monitor the presence of related antigen throughout the purification of JSRV.

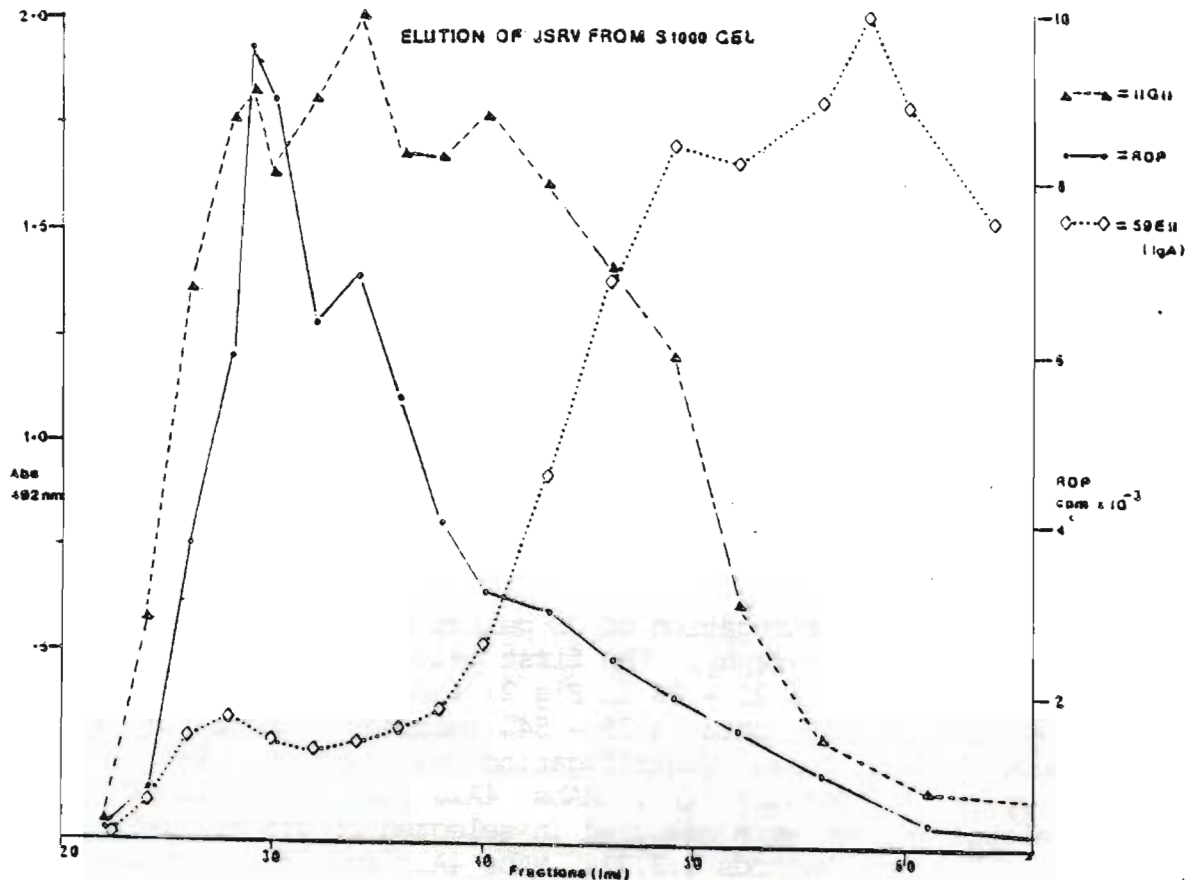
3.2.2.4.2 Monitoring of JSRV purification

The positive staining of antigens with the MAbs in JS tumour cell lines and frozen JS lung sections suggested that the MAbs were against antigens associated with jaagsiekte. As it was still not certain whether the antigens were of viral origin or associated with transformation it was decided to monitor the purification of JSRV from lung wash pellets using the MAbs in an ELISA and correlating the purification with the RDP assay results. There were two main objectives. Firstly, to determine whether the results of the

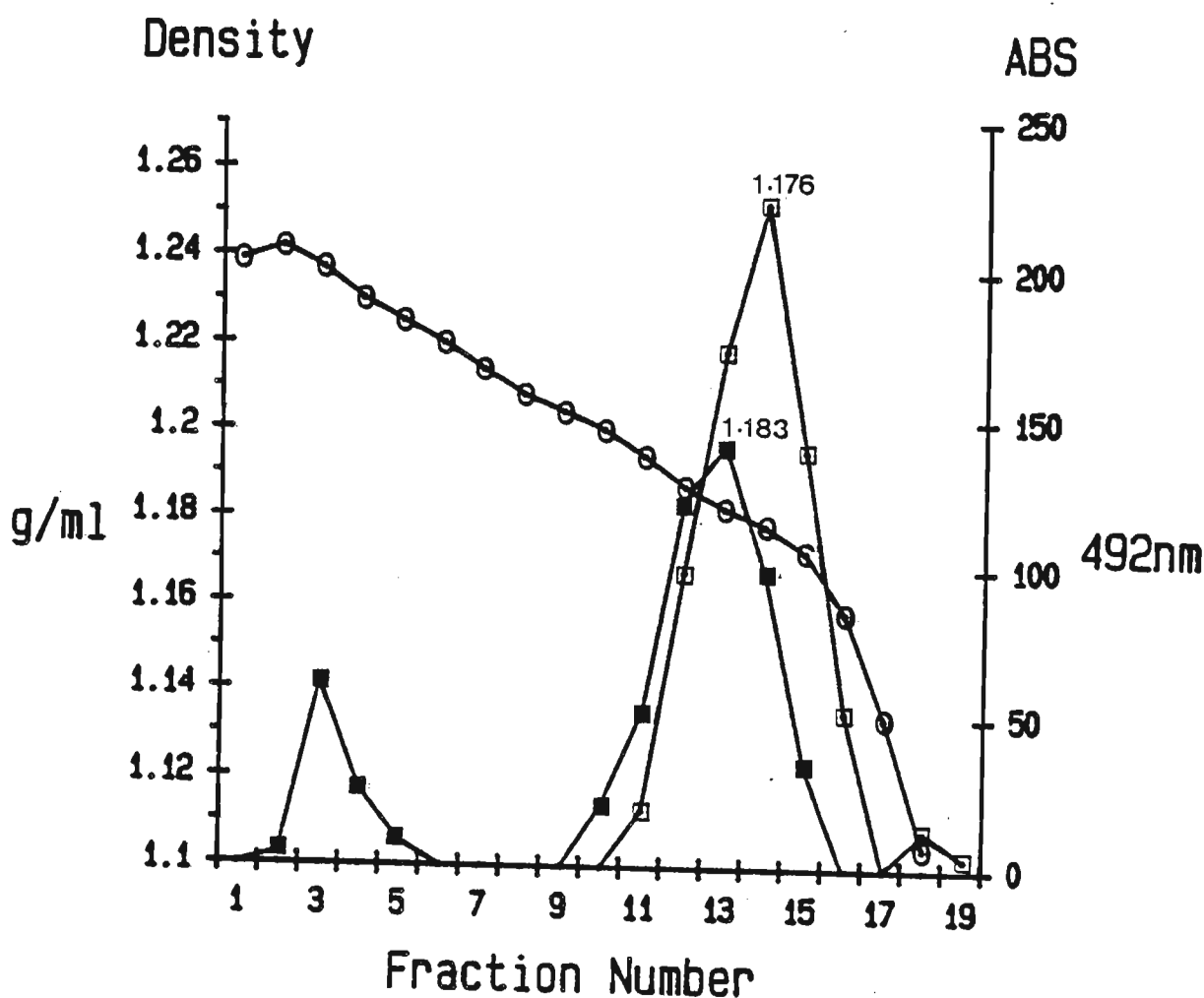
Fig 2. Gel chromatography of Freon extracted JS lung rinse pellet after treatment with 5 M urea for 1 min on Sephacryl S 1000 superfine gel. Sample: 4 ml; Column: Kl,6/40 (Pharmacia); Bed height: 32 cm; Eluant: buffer B; Flow rate 40 ml/h. (●) indicates RDP-activity; (▲) MAb 11G11 and (◇) MAb 59E11 related antigens were determined by means of an ELISA. MAbs and antigen were diluted 1:200 and 1:20, respectively. Absorbance values at 492 nm were used directly to indicate relative antigen concentration.

RDP-assay and the ELISA showed a correlation which would suggest that the MAbs are virus specific, and secondly, to determine whether the MAbs could replace the RDP assay in the monitoring of JSRV purifications.

Jaagsiekte infected lung pellets were treated with Freon, 5 M urea and passed through a Sephacryl S 1000 packed column. MAb 11G11 was used in the initial experiments together with MAb 59E11 in order to compare the elution of virus and IgA, a major non-viral contaminant in JS infected lungs. Urea was used to dissociate the immunoglobulins from the virus (see Fig. 2).



From this experiment it became clear that it would be difficult to determine conclusively whether the RDP and MAb 11G11-related antigen were from the same particle. The RDP would measure intact virus particles or cores which are in the first two peaks (Figure 2). MAb 11G11-detected antigen also peaks at these two positions, but there is also some of the antigen eluting later towards the free protein position. This is probably due to antigen forming part of various sub-viral particles and breakdown products. A useful result is the convincing separation of the IgA peak from the virus RDP peak. It was decided to repeat the experiment, combining the major fractions covering the area equivalent to the first peak and layering this on sucrose gradients. In this way the density of the particles, with which the MAbs reacted, could be determined (see Fig. 3).



The antigens with which MAb 2E3 and 11G11 reacted were consistently associated with particles at 1,176 g/ml in sucrose (MAb 2E3 values are not presented as they coincided exactly with those of MAb 11G11). MAb 4A10, on the other hand, reacted with a particle at a slightly higher density of 1,183 g/ml. This is very close to the density at which the RDP activity peaked (1,186 g/ml; see Fig 1). MAb 59E11, which is against IgA, reacts at the low density region where free protein would be found (not shown). From these results it was concluded that MAbs 2E3 and 11G11 react with a particle that has a slightly lower density than that of JSRV. MAb 4A10 on the other hand appears to be more JSRV-specific and it seems likely that it could be used to monitor the purification of JSRV. An important question still remained unanswered, namely: which were the proteins with which the MAbs reacted ?

3.2.2.4.3 Immunoblot

One of the ways to identify the antigens with which the MAbs react, is to use a Western blot technique, here referred to as the immunoblot technique. However, many repeated attempts, using this technique, failed to reveal any reaction between JS polypeptides and the 3 MAbs; 2E3, 4A10 and 11G11. To identify the reason for this a slight modification of the dot blot technique was used (see Methods 2.2.3). Briefly, JS antigen was dissolved in protein suspension buffer (PSB) of varying composition in terms of SDS and 2-mercaptoethanol and heated at 90 °C for 5 min. The treated samples were spotted onto nitrocellulose and overlaid with the MAbs. In Table 9 the result obtained when MAb 11G11 was used, is

presented.

TABLE 9 The effect of protein suspension buffer (PSB; see 2.8.2.1) and heat on the antigen with which MAb 11G11 reacts, determined using a dot blot technique (see 2.2.3).

Heat	Contents added to JS		Reaction with MAb
90 °C for 5 min.	PSB	+ 2-ME + SDS	- VE
	PSB	- 2-ME + SDS	+/-
	PSB	- SDS + 2-ME	- VE
	PSB	- SDS - 2-ME	- VE
No heat	PSB	+ 2-ME + SDS	- VE
	PSB	- 2-ME + SDS	+ VE
	PSB	- SDS + 2-ME	+ VE
	PSB	- SDS - 2-ME	+ VE

2-ME = 2-mercaptoethanol

Table 9 shows that heat and to a lesser degree 2-ME were the major reasons why the antigenicity was destroyed. For this reason a second experiment was done where the JS antigen was treated with PSB without 2-ME and heated at different temperatures for 5 and 120 min. The samples were spotted onto nitrocellulose and processed as described above (see Plate 10).

From the results shown in Plate 10 it is obvious that any heating, even at 37 °C, alters the antigenicity of the protein. For this reason, and as a last resort, a sample of the JS antigen was treated with PSB, without 2-ME, and separated on SDS-PAGE without heating the sample at all. A Coomassie stain of the gel showed that without heat and 2-ME, insufficient dissociation and separation of the viral proteins were obtained to allow the

min °C \ Ag		1/2	1/4	1/8	1/16	1/32	1/64	C
120	37							
	50							
	60							
	RT							
5	37							
	50							
	60							
	RT	○	○	○				

Plate 10 Dot blot result showing the effect of temperature on the antigenicity of MAb 11G11-related protein. Equal volumes of JS antigen and PSB, without 2-ME, were incubated at the indicated temperatures for 5 and 120 min. 100 ul was applied to each dot and developed as described in Methods (2.1.3). MAb 11G11 was used undiluted. A two-fold serial dilution of the antigen was used. C, represents conjugate control.

determination of the size of the MAb-related antigen. Transfer of the proteins from this gel to nitrocellulose and subsequent overlay with MAb 11G11 identified a smear between the stacking and separating region of the gel, confirming a lack of separation. Similar results were obtained when MAbs 2E3 and 4A10 were used. Attempts to use an urea-containing gel system were also unsuccessful.

3.2.2.4.4 Affinity chromatography

An alternative approach to identify the proteins with which the MAbs react is to use affinity chromatography. For a reason not yet established, success was only achieved using a MAb 11G11 affinity column. Extracts of both JS tumour cell lines, 15.4 and 21.3, were passed through the 11G11 affinity column. The antigen that was retained has a molecular weight of approximately 50 000 dalton on SDS-PAGE and is called p50 (see Plate 11). This result was also obtained when the tumour cells were labelled with ³⁵S-methionine.

A little disturbing, but in agreement with previous results, was the finding that an identical protein was purified from labelled normal type II cells. This finding supports the previous suggestion that MAb 11G11 reacts with a normal protein that is produced in larger amounts in transformed cells.

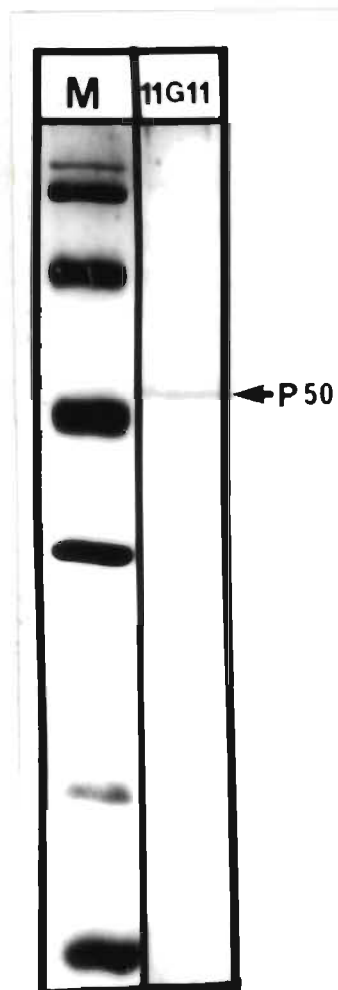


Plate 11 SDS-PAGE of the protein eluted from a MAb 11G11 affinity column. Lane 11G11 shows the protein (p50) which was purified from soluble extracts of a JS tumour cell line using MAb 11G11 bound to CNBr activated Sepharose 4B (see 2.6.2). Lane M represents low molecular weight markers (Pharmacia, Uppsala, Sweden).

3.2.2.4.5 Screening of a JS lesion DNA library

A library was made of DNA extracted from a JS lesion. The DNA was digested, sized and cloned into gtl1 as described under Methods (2.11). The aim was to use the MAbs to identify clones containing the gene coding for the related antigen. It was hoped that this sequence could be used, inter alia, for a JS-specific diagnostic probe, to determine whether the JSRV virus is endogenous and to isolate sequences slightly larger than itself and "walk the genome" so that ultimately the total JSRV genome could be sequenced.

More than 25 000 plaques were screened with a cocktail of the 3 MAbs (see Methods 2.12). However, no plaques were found producing a MAb related protein. It was therefore decided to discontinue screening the library, which obviously contained a high percentage of normal sheep DNA. Instead, it was decided to adopt a more direct and specific approach to clone and screen the cDNA of JSRV RNA.

3.2.2.4.6 Screening of a JSRV cDNA library

JSRV purified according to the protocol described under Methods (2.13) was found to be relatively free from most cellular RNA. RNA was therefore extracted from purified JSRV and cDNA was synthesized as described in 2.14. The cDNA was then cloned into pUC 13 in this laboratory by Elsa Posnett, as part of a collaborative project. Recombinant plasmids were transfected into E. coli JM105

in order to screen for the proteins coded for by the inserts with the MAbs (see 2.15).

One of the clones screened was shown repeatedly to produce a protein that reacted strongly with MAb 4A10. This clone is referred to as JS4 (see Plate 12). This was a most exciting result and is presently being further investigated by my colleague. It is hoped that this clone and MAb may prove to be the breakthrough that has been so long awaited in JS research.


M Ab'S				
CLONES	2E3	11G11	4A10	C
4				
59				
66				
C				

Plate 12 Detection of MAb 4A10-related antigen produced by clone JS4 using an indirect immunoperoxidase overlay technique (see 2.15.3). Transformed colonies were lysed as described in Methods (see 2.15) and overlaid with undiluted MAb culture supernatants. Under heading CLONES, row C represents pUC 13 without any insert and transformed into E. coli JM105. Under heading MAb's, column C, represents mouse conjugate control.

3.2.2.5 Polyclonal antibodies

A major limitation of the JS specific MAbs was that they did not react in an immunoblot technique. This seems to be a common problem with those MAbs that are initially screened using an ELISA technique (Steineman, Fenner, Binz and Parish, 1984). Although MAbs are considered to be specific, results obtained using an ELISA are often less satisfactory than those obtained by an immunoblot technique. A case in point is the consideration of the immunoblot as the golden method to finalize whether a serum has antibodies to the AIDS virus.

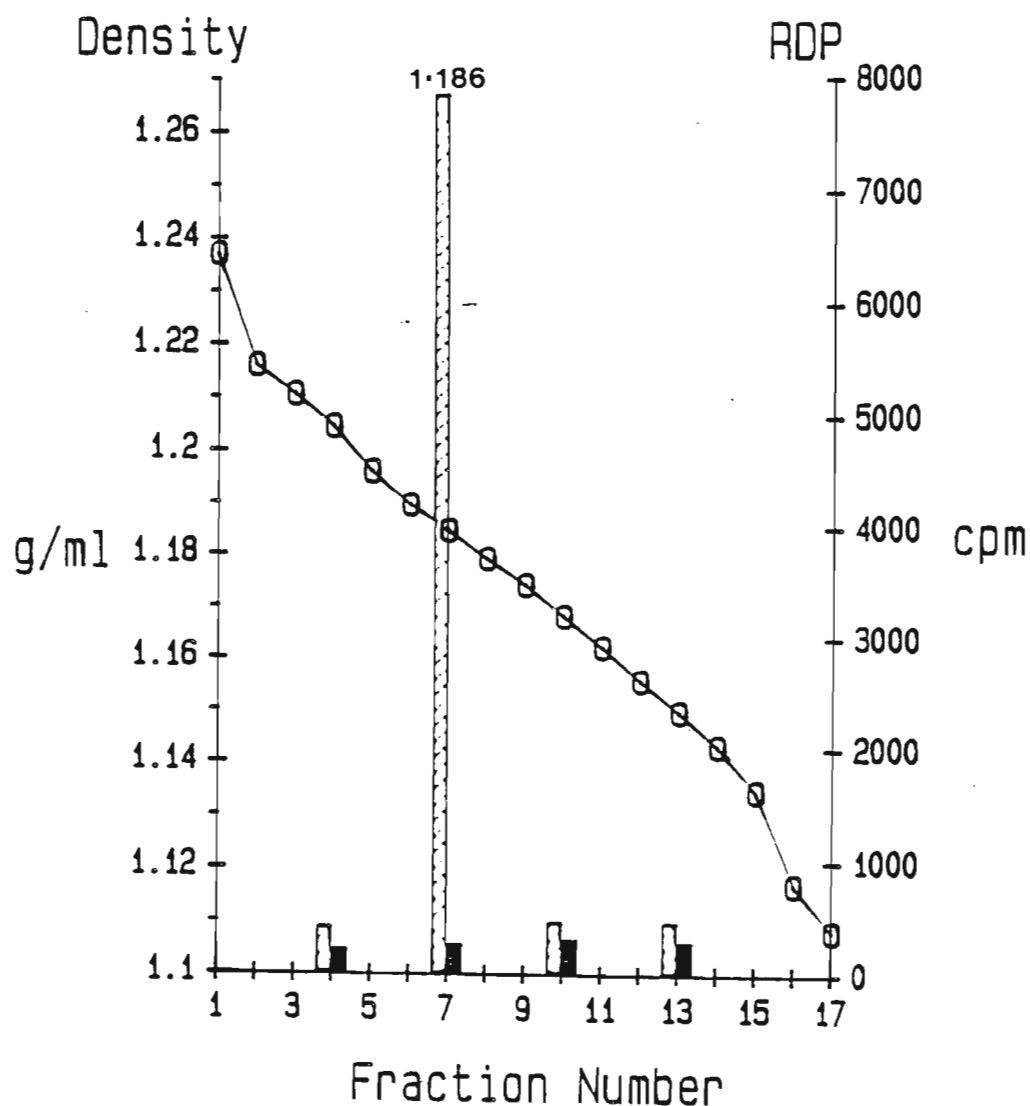
For the author's M.Sc. thesis a polyclonal antiserum was made in rabbits against "purified" JSRV antigen. However, this serum, although it contains antibodies against a few JSRV proteins, also reacts with many non-viral proteins, especially immunoglobulins. This was the case even after the serum had been extensively absorbed with normal lung material.

A more useful polyclonal serum was found by Sharp and Herring in 1983 when they showed that serum against the group-specific protein of MPMV cross-reacted with JS lung material. It has proved most useful in JS research when used in an immunoblot technique. Plate 13 shows the result obtained when MPMV (p27) antiserum was reacted with both JS and normal lung wash pellets. Note the absence of any reaction with the normal lung material and the size of the JS protein with which the polyclonal serum reacts. This protein is referred to as the p29.



Plate 13 An immunoblot result showing the protein detected by the goat antiserum to MPMV (p27) in JS material but not in normal lung material. Lane J1, represents JS lung rinse material and lane NL, normal lung material. MPMV antiserum was used at 1:400 dilution. The procedure used was as described in 2.8.2.2.

The immunoblot technique using MPMV antiserum was also used in parallel with the RDP assay to monitor the purification of JSRV. From the MAb work, it was realized that more information was obtained if the virus was monitored when purified by isopycnic centrifugation rather than column purification. In Figure 4 it is seen that the RDP activity peak fractions coincide with those fractions that react most intensely with the MPMV antiserum on the blot (Plate 14). This result was most encouraging as it supported the contention that the MPMV antiserum reacts with a JSRV-specific protein. The importance of this reaction will become evident in the next two chapters.



[illegible]

Plate 14 An immunoblot result showing the density of the JS p29 antigen when JS lung rinse pellet was centrifuged to isopycnic equilibrium and its absence in normal lung rinse pellets (see Fig. 4). Samples (30 ul) representing groups of three fractions (as indicated in the legend to Fig 4) were separated on a 12,5% SDS-PAG (see 2.8) and transferred to nitrocellulose (see 2.8.2). The transferred proteins were overlayed with MPMV anti-serum (1:400) and the reaction developed as described in 2.8.2.2. Note that staining only occurred in group 2, which has a density around 1,186 g/ml. There was no staining in the normal lung fractions.

3.2.3 Morphology of JSRV

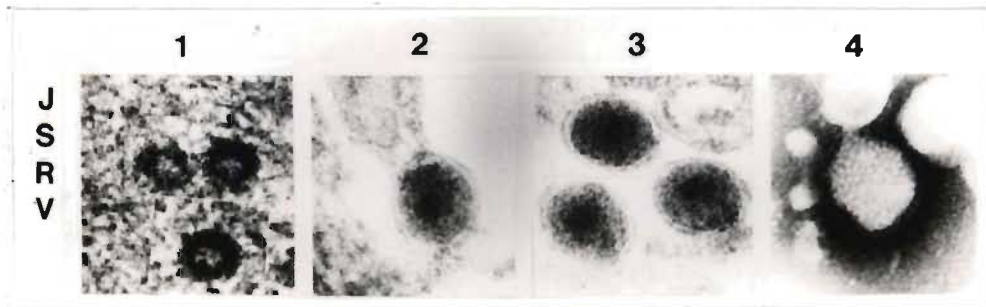
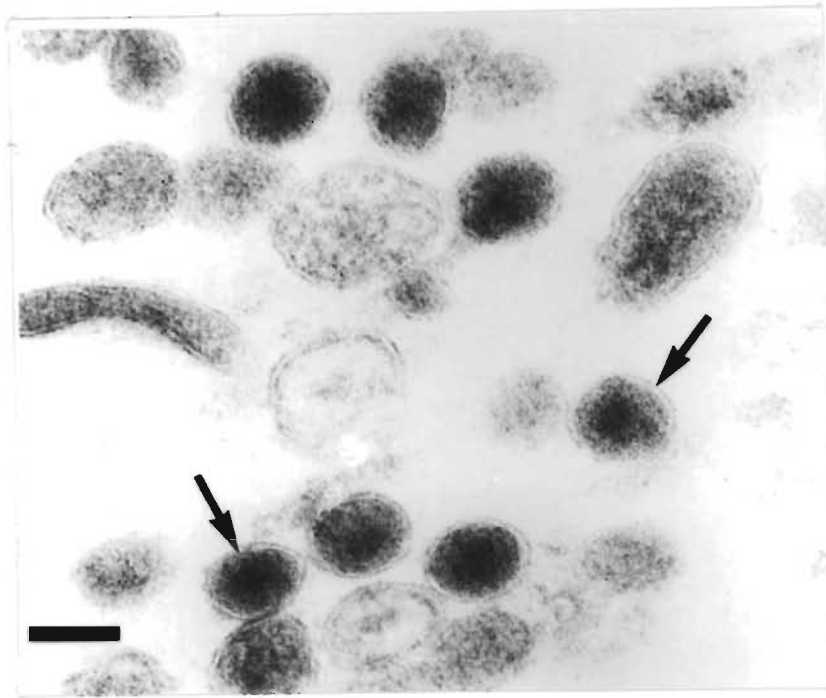
Samples taken from the JSRV 1,186 g/ml density peak region were observed in an electron microscope. Particles were observed, as shown in Plate 15, having a unit membrane and an eccentric, round nucleoid. The perinucleoidal space was electron dense and the core was always visible as a more electron dense spot in a slightly eccentric position.

Plate 16 shows the different stages of JSRV maturation. In column 1, the intracytoplasmic JSRV particles are seen. They undergo a maturation process at the plasma membrane (column 2) and bud to form mature particles (column 3). Plate 16, column 4, represents negatively stained JSRV showing the surface spikes.

Plate 16 has been included so that the morphogenesis of MVV and BRV can be compared with that of JSRV. These will be discussed in chapter 6.

Plate 15 The morphology of mature isopycnicallly purified JSRV taken from the 1,186 g/ml density fraction where the RDP and p29 peak. Bar represents 100 nm.

Plate 16 Transmission electron microscopic observations of the different stages of JSRV maturation. Column 1, shows the intracytoplasmic JSRV particles. Column 2, budding, column 3, mature and column 4, negatively stained JSRV particles showing the surface spikes. This plate was kindly provided by A. Payne (Payne, 1985).



3.3 DISCUSSION

Jaagsiekte retrovirus has been purified to an extent such that its polypeptide composition could be determined. These proteins are denoted p26; p29; p32; p38; p50; p75-p77; p81 and p84 (see Plate 2). Evidence was presented in the author's MSc thesis which supports the view that p26; p29; p32 p81 and p84 are JSRV polypeptides. It was suggested that p50 is a normal lung protein. The polypeptide pattern presented in Plate 2 has been repeatedly observed in more than 200 JS purifications that were monitored on SDS-PAGE. Emphasis has been placed on the polypeptide pattern in order to identify JSRV and to be able to compare its polypeptide pattern with that of other viruses.

The conclusion drawn when the JSRV polypeptide pattern was compared with those of other known retroviruses was that there was little similarity, except for the BRV polypeptide pattern which will be discussed in Chapter 5. The main difference between JSRV and the other oncoviruses was an absence of low molecular weight proteins, viz. p10; p12 and p15. This could be explained by the absence of a cleavage mechanism that is present in the other viruses (Verwoerd et al., 1983). Attempts to stain the glycoproteins were unsuccessful, because of the presence of immunoglobulins which were evident even in the best virus pre-parations. Our inability to cultivate virus in vitro also restricted the identification of phosphoproteins and glycoproteins using isotope labelling techniques. Nevertheless, the protein pattern is clear and consistent enough to allow comparison to other viruses

associated with JS.

One of the major motivations for the production of MAbs was their possible usefulness in sorting out the proteins of JSRV. It was unfortunate though, that none of the MAbs reacted in an immunoblot technique. However, this appears to be a common problem with MAbs that are initially screened using non denatured antigen (Steineman et al., 1984). It was shown that heating was the major reason for the loss of antigenicity and attempts to separate the proteins using urea gels, to avoid the heating problem, were also unsuccessful.

The observation that MAbs 2E3 and 4A10 did not bind any protein when bound to CNBr affinity columns was also disappointing. However, here again it is a known problem with some MAbs that the chemical coupling of the MAb to the insoluble support often results in an alteration of the antigenic binding site and hence alters their specificity (Johnstone and Thorpe, 1982). Nevertheless, MAb 11G11 bound a p50 protein. This protein was previously considered to be a normal protein, possibly incorporated into JSRV (York, 1984). In agreement with this hypothesis was the finding that p50 could also be purified from labelled normal lung cells using the same affinity column.

Both MAbs 2E3 and 11G11 reacted with a low but significant number of normal lung preparations. The staining in the normal lungs was always shown to be localized to the bronchiole (Plates 7b and 8b). In 1986, Endo, Kamma and Ogata reported that MAbs were not as

specific as they were previously thought to be. They isolated two murine monoclonal antibodies against human lung cancer antigens. One of the MABs (MAB 8) was shown to bind to 68% of the 65 lung cancers tested. This MAB also reacted with 4 lung bronchiole sections out of 9 normal lungs. Their MAB 8 recognized a protein of molecular weight 48 000 dalton. A virus has not yet been implicated in the etiology of human lung cancer, so there was no speculation of this protein being a viral protein. Numerous other reports also reveal that MABs can be non-specific (Embleton, Habib, Garnett and Wood, 1986).

It has also been shown that monoclonal antiviral antibodies often react with normal tissue (Fujinami, Oldstone, Wroblewska, Frankel and Koprowski, 1983). Srinivasappa, Saegusa, Prabhakar, Gentry, Buchmeier, Wiktor, Kaprowski, Oldstone and Notkins (1986) showed that more than 3,5% of the antiviral MABs they tested reacted with normal tissues. This high yield is not surprising when one considers that an antibody is capable of recognizing an antigenic determinant as short as four to six amino acids (Atassie, 1975). Since there are only 20 common amino acids and assuming that these amino acids are randomly distributed, it has been estimated that a given sequence of four amino acids would appear once in every 20^4 tetramers, five amino acids once in every 20^5 pentamers and a given sequence of six amino acids once in every 20^6 hexamers (Crawford, Leppard, Lane and Harlow, 1982). Thus, considering the tens of thousands of proteins in an organism, the probability of a similar sequence appearing by chance in two unrelated molecules is not insignificant. This may explain why

MABs 2E3 and 11G11 reacted with normal tissue in the bronchiole.

There is a possibility that MAb 11G11 may be against an onco-protein since it stains JS infected lung sections with much greater intensity than normal lungs. MAb 2E3 appears to react in a very similar manner to MAb 11G11 and they both react with the same particle at a density of 1,176 g/ml (Figure 3). Although they belong to the same subclass they are not considered to be identical, but rather to react with different epitopes of the same protein. This is supported by the results of competition studies using an ELISA (results not shown) indicating that they do not compete for the same epitope. In addition, the ability of MAb 11G11 against the inability of 2E3 to bind protein when used in affinity chromatography also indicate that they are not identical MABs.

MAb 4A10 on the other hand appears to be a more JSRV-specific antibody than 2E3 and 11G11. The absence of staining of any normal lung frozen sections (Plate 4b) as opposed to the very specific and intense staining of JS lung sections (Plate 4a), strongly suggests that MAb 4A10 is JS-specific. MAb 4A10 also stained a low percentage of the cells in 15.4 and 21.3 JS tumour cell lines (Plate 8a). This is in agreement with the RDP activity results which also suggest that only a low percentage of the cells are producing JSRV (Verwoerd, unpublished results). The presence of JSRV in the transformed cells has been confirmed by infectivity studies (Coetzee et al., 1976). It is most useful that MAB 4A10

reacted with JS tumour cells on sections that had been fixed in formalin and embedded in paraffin. Unfortunately, not all the tumour cells in all the lung sections examined stained up. This would have been most useful in confirming the pathologist's diagnosis of JS. On the other hand, this result is in agreement with the suggestion that MAb 4A10 is against a viral protein, as it is believed that not all tumour cells are actively producing virus.

That none of the plaques, containing inserts of JS lesion DNA, reacted with any of the MAbs was a little disturbing. However, the presence of a vast excess of normal sheep DNA in such a library reduced the probability of detecting a JS-specific sequence to such an extent that it was decided to rather screen a cDNA library. It was therefore decided that the chances would be increased if cDNA of JSRV RNA was cloned and screened for expression.

The availability of a very good cDNA synthesis kit made the production of JSRV cDNA from JS mRNA relatively easy. This kit required very little RNA and the complete synthesis was done in a single Eppendorf tube. This meant that there was no transferring of the viral RNA during the production of the cDNA, therefore losses due to handling were minimized. This was an important consideration as the supply of JSRV RNA was very limited.

The reaction of MAb 4A10 with the JS4 clone is most promising. If this clone proves to be JS-specific by means of hybridization studies it will confirm that MAb 4A10 is JSRV-specific. This

result will have many useful applications in the JS project. For example, it will be possible to determine whether JSRV is endogenous or not, and the development of a diagnostic probe could become feasible.

The use of MPMV (p27) antiserum and an immunoblot technique to identify JSRV was a major breakthrough in the JS research. The demonstration that the most intense staining of JSRV p29 (Plate 14) correlated with the RDP peak fractions (Figure 4; fraction 7), when JSRV was isopycnically purified, was convincing confirmation that the p29 is a JS protein. This serological cross-reaction between the group-specific antigen of MPMV and the p29 of JSRV is most intriguing as it is the same serological reaction that links JSRV to BRV. The latter will be discussed in greater detail in chapter 6.

The ultrastructure of JSRV has been compared with the members of the type B (MMTV), type C (MuSV), type D (SMRV) as well as BLV (Payne et al., 1983). The conclusion drawn was that JSRV differs from the other retroviruses by its relatively dense perinucleoidal space, its slightly dense nucleoid and its surface spikes. It should thus be considered in a class of its own. The morphological features will be further discussed in the final chapter.

In summary, the biochemical features of JSRV are that it has RDP activity, a buoyant density in sucrose of 1,186 g/ml and has eight polypeptides ranging in molecular weights between 26 000 and 84

000 daltons. The immunological characteristics of JSRV include a serological relationship to the prototype D and B retroviruses. In addition JS-specific MAbs have been isolated, one of which detects the p50 protein, which could be an oncoprotein as it is produced in increased amounts in JS-infected lungs. MAb 4A10, which is regarded as being JSRV-specific, reacts with a clone that was constructed from cDNA of purified JSRV RNA. It also appears that this MAb could be useful in the development of a JS diagnostic assay.

A knowledge of the biochemical, immunological and morphological characteristics of JSRV is very important, especially in the case of the JS disease complex where other viruses are also found in the same infected animal. With the techniques developed and discussed in this chapter it was possible to easily identify and distinguish JSRV from other retroviruses. In the following chapter an investigation of a second retrovirus, which is often associated with JS infected sheep, will be presented.

CHAPTER 4

THE LENTIVIRUS (SA-OMVV)

4.1 INTRODUCTION

The role of the lentivirus in JS is uncertain. Until very recently, Maedi-Visna Virus (MVV), the prototype of the family Lentiviridae, was not considered to be present in South Africa (Payne et al., 1986). Because of its assumed absence in this country its possible involvement in JS was not considered. However, approximately 3 years ago this laboratory isolated a virus from cell cultures that had been infected with lung rinse material from a jaagsiekte-infected sheep. Further investigations revealed that this virus was related to Maedi-Visna Virus. This finding initiated the search for a test whereby sheep sera could be screened for the presence of antibodies to the virus.

As there were many problems associated with the ELISA, the Western blot technique was adopted to screen sheep sera sent in from farms throughout the country, as well as sera from our experimental sheep, for MVV antibodies. The results revealed a high incidence of antibodies to MVV, suggesting that the sheep were, or had been, infected with MVV. Furthermore, MVV could be isolated from nearly all macrophage cultures established from JS infected sheep (Payne et al., 1986).

This finding suggested that the lentivirus might also be present in JS lung rinse material, our only source of JSRV. MVV, being a

retrovirus, would also be detected with the RDP assay, which, as mentioned in chapter 3, was for a long time the only method used to detect JSRV. Its presence could therefore lead to false positive results as far as JSRV was concerned. Fortunately, this has changed and it is now also possible to monitor JSRV using an immunoblot technique as described and discussed in the previous chapter.

In this chapter the use of the immunoblot technique to differentiate between JSRV and MVV is presented. Using this method, and MPMV and MVV anti-sera, it is shown that both JSRV and MVV are present in the same lung rinse pellet. The co-purification of MVV with JSRV and the results of an investigation into the effect of Freon on MVV are also presented.

The MVV-isolate referred to in this chapter was sent over to Dr R. Vigne (a world authority on MVV) for further characterization and comparison with the type strain of MVV, which is the Icelandic strain K1514 (Querant, Barban, Sauze, Vigne, Payne, York, DeVilliers and Verwoerd, 1987). Based on differences in restriction maps, his group showed that the South African isolate of MVV was different from the type strain of MVV. This isolate has been called SA-OMVV I (South African Ovine Maedi-Visna virus type I) (Payne et al., 1986). From now on the isolate worked with in this thesis will be referred to as SA-OMVV I. MVV will be used to refer to the prototype strain.

From the investigations into the presence of SA-OMVV I in JS lung

pellets, presented in this chapter, the biochemical, immunological and morphological characteristics of the virus also become apparent. These will be highlighted in the discussion of this chapter.

4.2 RESULTS

4.2.1 Immunoblot to differentiate between JSRV and SA-OMV I

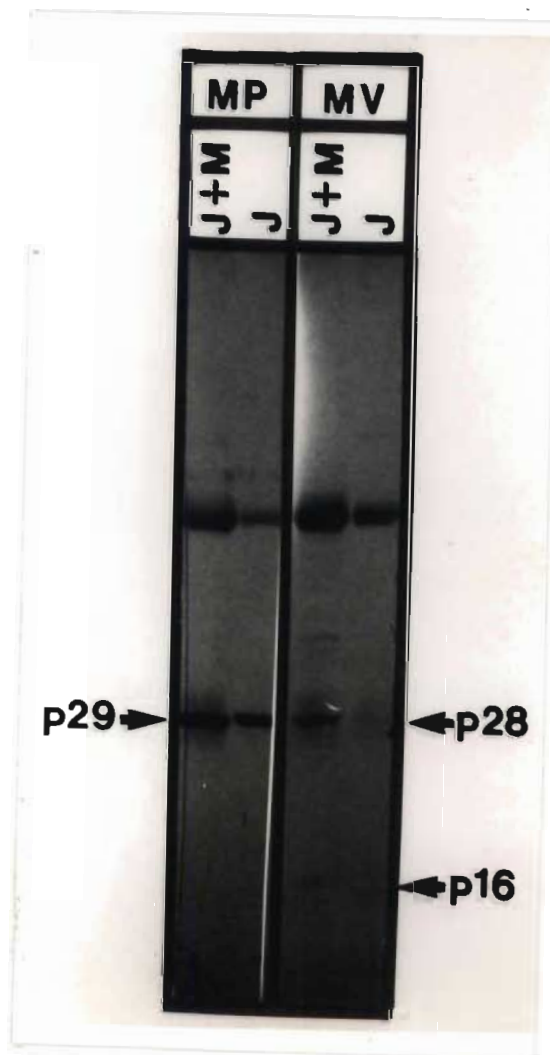
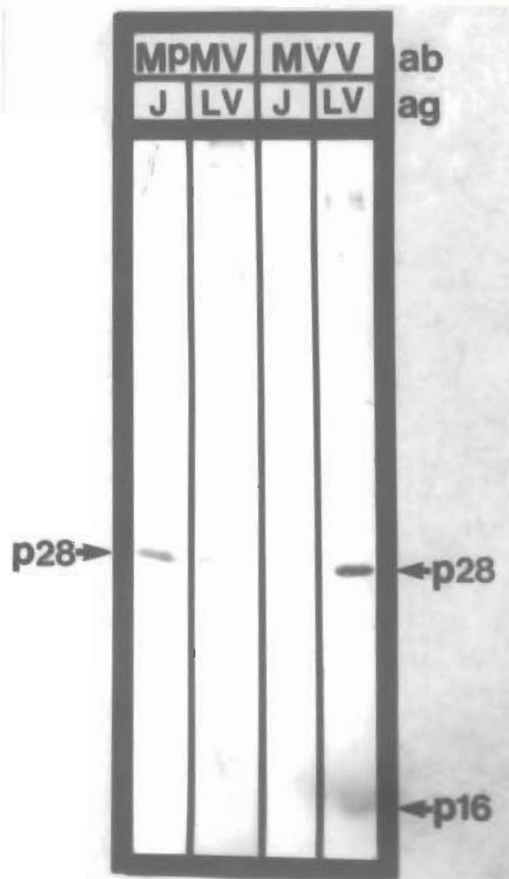
As mentioned earlier, the ELISA was not satisfactory as a method to detect antibodies in sheep sera. Too many false positives were obtained (personal observations). For this reason use was made of the immunoblot technique. Although it is more costly and time consuming than the ELISA, it is a very valuable method. In Plate 17 it is shown that MVV antiserum react with two antigens present in the LV lane. These two proteins detected by MVV antiserum are p28, the group-specific antigen of the lentiviruses, and p16, the type-specific antigen of MVV (Matthews, 1982). In lane LV under the MPMV heading no bands are seen, whereas in lane J, a similar sized band to the LV p28 is detected. This technique and the two antisera, viz. MVV and MPMV, were used throughout the JSRV purifications to detect which virus or viruses were present in the lung wash pellet being purified.

4.2.2 Detection of both JSRV and SA-OMV I in the same lung wash pellet

As a standard procedure RDP activity assays were done on JS lung wash pellets. This was to quantitate JSRV. In the past it was thought that only one retrovirus was present and that any RDP activity was due only to JSRV. However, with the above mentioned technique it was now possible to determine whether MVV was also present. In Plate 18 it is clearly shown that both MVV and JS proteins are detected in the same pellet.

Plate 17 An immunoblot result showing the polypeptides of SA-OMVV I and JSRV which react with MVV and MPMV antisera, respectively. Heading MPMV, represents goat antiserum to MPMV against JSRV (lane J) and SA - OMVV I (lane LV) polypeptides. Heading MVV, represents sheep antiserum to MVV against JSRV (lane J) and SA - OMVV I (lane LV) polypeptides. MPMV and MVV antisera were used at 1:400 and 1:50 dilutions, respectively.

Plate 18 An immunoblot result showing the presence of both SA-OMVV I and JS antigen in the same pellet. Lane J+M, represents a lung wash pellet from a sheep lung infected with JS. Under heading MPMV, lane J+M shows JS protein p29. Lane JS represents lung wash pellet from a sheep lung infected with JS but free of SA - OMVV I. Under heading MV, lane J+M reveals the two SA - OMVV proteins, viz. p28 and p16. The two proteins are absent in lane JS. The top band is a non - specific reaction from the conjugate.



The finding that SA-OMV I was present in some JS lung wash pellets meant that all pellets were thereafter tested for its presence as a standard procedure. JSRV purifications were subsequently also monitored using the immunoblot technique and both MPMV and MVV antisera. Some interesting observations were made.

4.2.3 Purification of lung wash pellets containing both JSRV and SA-OMV I.

4.2.3.1 Isopycnic fractionation of JSRV/SA-OMV I lung pellet without Freon treatment

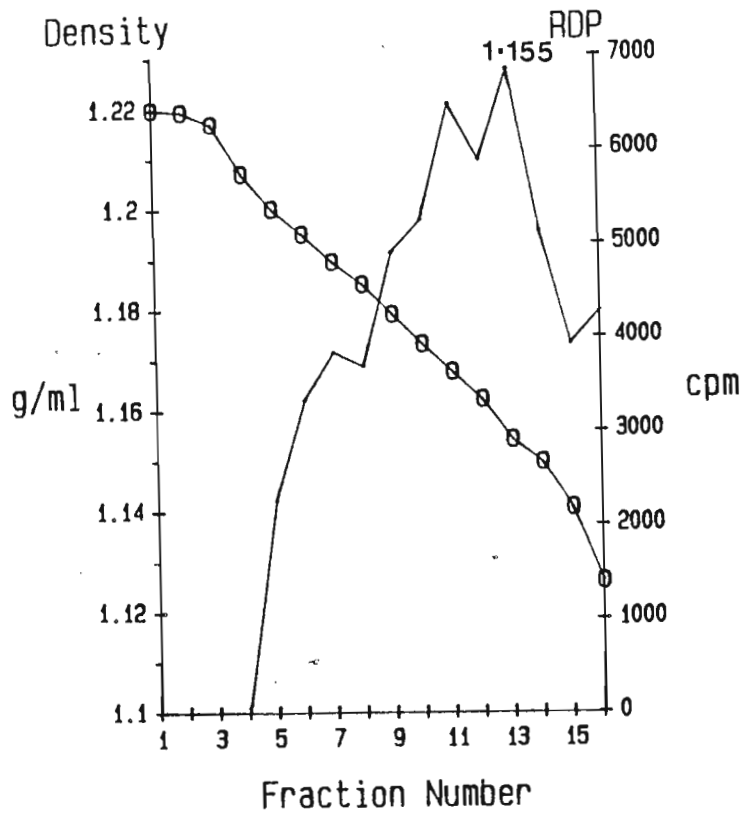
A lung wash pellet from a JS infected lung, known to contain both JSRV and SA - OMV I, was layered onto a sucrose gradient and centrifuged to equilibrium (Fig. 5). Fractions were monitored for RDP- activity, JS and SA - OMV I presence (using an immunoblot technique) and observed using the TEM (see Table 10).

TABLE 10: Transmission electron microscopic (TEM) observations of selected negatively stained samples of pellet 6691 (without prior Freon treatment) after isopycnic centrifugation. The fraction numbers are identical to those illustrated in Fig. 5a.

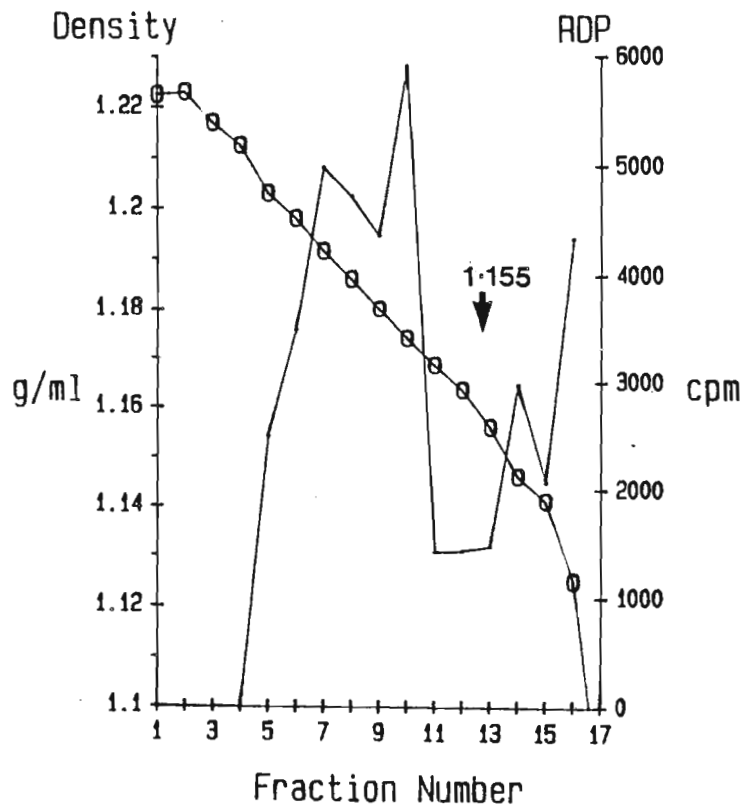
Frac. No	Density	TEM			BLOT	
		Virus	Amount	Comments	JS	MVV
6	1,195	JSRV	++	mostly cores	+	-
7	1,189	JSRV	+++	mostly cores	++	-
8	1,186	JSRV	++++	whole virus	++++	+
9	1,179	JSRV	+++	surface structure not too clear	+++	++
10	1,173	JSRV/MVV	++	some JSRV but mostly smooth MVV	++	+++
11	1,167	MVV	+++	mostly smooth	+	++++
12	1,162	MVV	+++	only smooth	-	+++
13	1,154	MVV	+++	only smooth	-	+++

+, under blot represents intensity of the band; +, under amount refers to the concentration of viral particles observed. One +, is low and four +, is high. -, represents negative.

a



b



The electron microscopic observations agreed with the blot results (Plates 19a and b). These have been included in Table 10 to make the comparative observations easier. TEM observations confirmed that JSRV was at the higher 1,186 g/ml fractions and SA - OMV I at the lower densities 1,154 - 1,173 g/ml. The TEM observations of fractions after the pellet was treated with Freon (not presented here), revealed firstly that JSRV was much cleaner than without Freon treatment and secondly that very little SA - OMV I was observed in the lower density fractions (Fig. 5b; 1,155 g/ml region). This was in agreement with the blot results (see Plates 19 and 20).

Without prior Freon treatment two RDP peaks are observed, one at 1,168 g/ml and a second at 1,155 g/ml (Fig. 5a). These two peaks represent SA - OMV I, from the blot results (Plate 19b) and TEM observations (Table 10). There is also a peak at 1,19 g/ml (Fig. 5a) but from TEM this peak contains cores. It is impossible to differentiate between MVV and JSRV cores using negatively stained samples. However, the blot result indicates that they are JSRV cores (Plate 19a). There is a slight shoulder at fraction 8 (Fig. 5a) which is the JSRV RDP peak. The blot results and TEM observations support this. It is therefore clear that both JSRV and SA - OMV I are present in the same pellet but can be separated by means of their densities.

In Fig. 5b (after Freon treatment), note that the RDP peak around the 1,155 g/ml density region is absent but that there is still RDP activity and SA - OMV I antigen (Plate 19b) at the slightly

Plate 19a An immunoblot result showing the fractions which contain the p29 antigen when pellet 6691, containing both JSRV and SA - OMV I, was centrifuged to isopycnic equilibrium (see Fig. 5a). Lane M, represents low molecular weight markers. Anti-MPMV (p27) serum was used at 1:400 dilution. Note that fraction 8 has a density of 1,186 g/ml and the absence of any reaction in lanes 11-16.

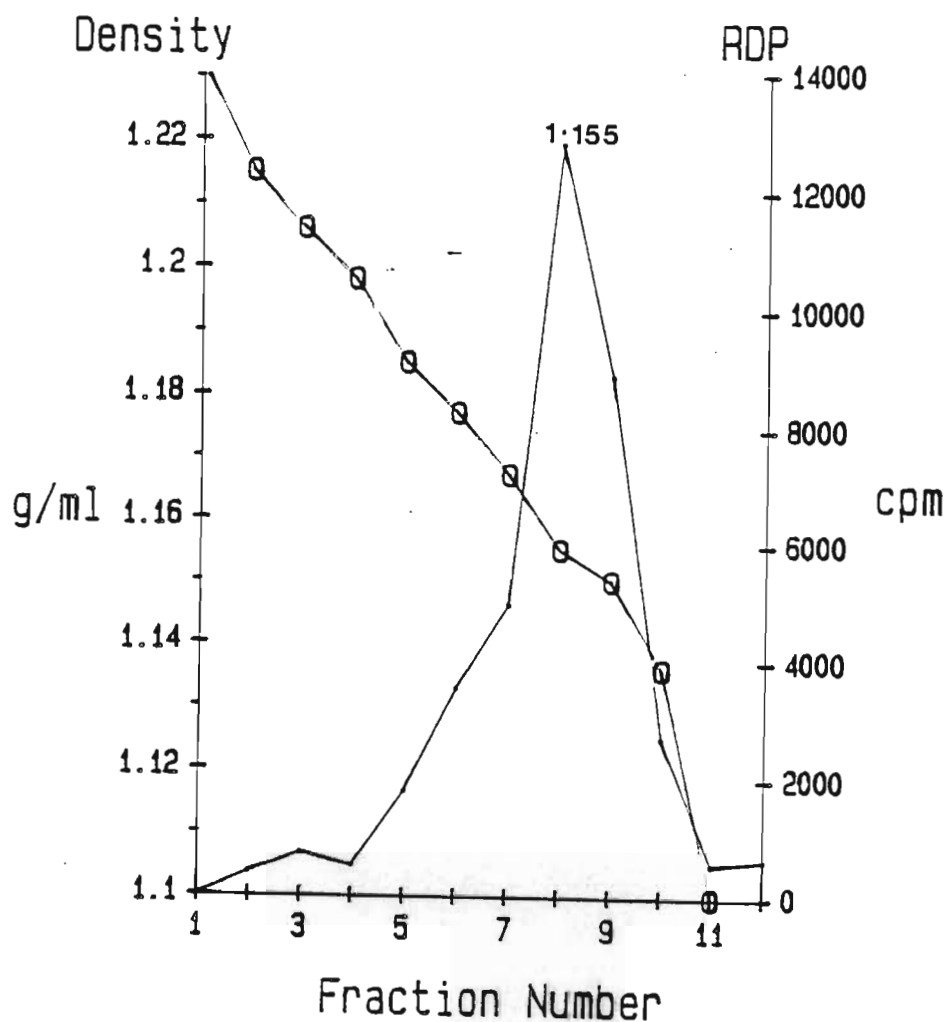
Plate 19b An immunoblot result showing the fractions which contain the p28 SA - OMV I antigen. Equivalent samples were used as in the above plate. In this blot anti-MV serum was used at 1:50 dilution. Most of the SA - OMV I antigen is found in fraction 11 which has a density of 1,168 g/ml. Note the very faint reaction in lanes 7 and 8. The plates show very clearly the presence of both JSRV and SA - OMV I in the same pellet.

Plate 20a An immunoblot result showing the effect that Freon had when pellet 6691 was centrifuged to isopycnic equilibrium as described in the legend to Fig. 5b. JSRV p29 antigen was detected using MPMV anti-serum diluted at 1:400. Note that fractions 7 and 9 contain all the virus. The peak density for these two fractions is at 1,186 g/ml.

Plate 20b An immunoblot result when the same fractions used in Plate 20a were overlayed with anti-MVV serum to detect SA - OMVV I antigen. The most intense reaction is seen at fraction 9 which has a density of 1,18 g/ml. Note the absence of the band in fractions 11 - 15.

higher density of 1,18 g/ml, although in much lower concentrations than before Freon treatment. The reason for this is uncertain.

The possibility that SA-OMVV I from an in vivo source (sheep lungs) might differ from in vitro cultured SA - OMVV I was suggested. To investigate this, the South African strain of in vitro cultured MVV was sedimented to isopycnic equilibrium and monitored using the RDP-assay, so as to determine its density and compare its RDP profile with in vivo produced MVV. In Fig. 6 it is shown that in vitro cultured MVV bands as a single peak at a density of 1,155 g/ml. As shown in Fig. 5, in vivo produced MVV has two density peaks at 1,155 and 1,168 g/ml. The possible nature of the latter peak is discussed later.



Although Freon treatment significantly reduced the concentration of SA-OMVV I, Freon was also shown to reduce the concentration of the JSRV. In an attempt to remove SA-OMVV I from JSRV, without Freon treatment, it was decided to adjust the gradient conditions to suit the JSRV purification. The results and gradient conditions used to separate these two viruses are presented in Fig. 7.

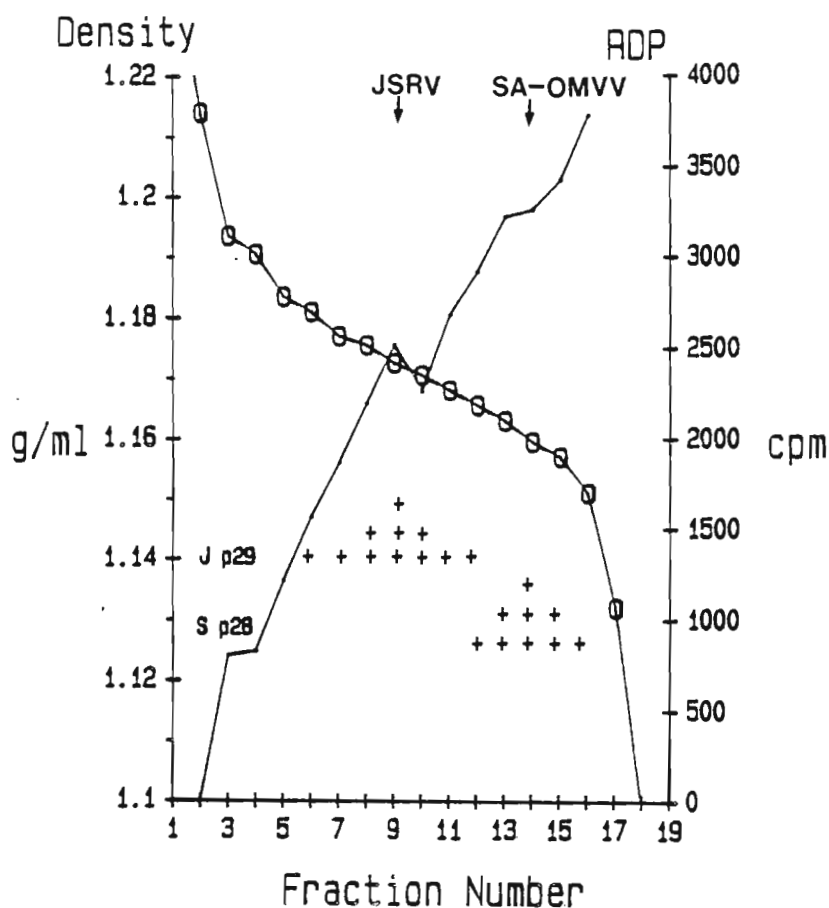


Fig. 7 Isopycnic centrifugation of pellet 6691, containing both JSRV and SA - OMVV I, without Freon treatment, through a sucrose gradient. The pellet (2 ml) was layered on a 30 - 40 % (w/v) sucrose gradient in a Beckman SW 27 tube. Centrifugation was overnight (85 000 x g; 4 C). Density (O) and RDP activity (●), were measured in selected fractions. Fractions were diluted and pelleted as described in the legend to Fig. 5. Samples were separated on SDS - PAGE and immunoblotted. MVV and MPMV antisera were used to detect SA - OMVV I (p28) and JSRV (p29), respectively. The intensity of the two proteins are indicated with +'s. One + represents 1/3 of the positive control which is taken as 1.

In Fig. 7 it is shown that by changing the gradient concentrations from 20 - 50 % to 30 - 40 % (w/v) sucrose it was possible to separate JSRV and SA - OMVV I from each other. Although the RDP activity curve does not indicate separation, the immunoblot results show that the lentivirus position is shifted significantly towards the lower density region. Fraction 7, 8 and even 9, which contain a lot of JSRV p29 are shown to be relatively free of SA-OMVV I p28.

4.2.4 Polypeptide pattern of SA - OMVV I

It was mentioned in the introduction to this chapter that the South African isolate of MVV was shown to be genetically differentiated from the Icelandic type strain of MVV (Payne et al., 1986). It was therefore of interest to see whether a comparison of the polypeptide pattern of SA - OMVV I with that published for MVV (Icelandic strain) would support this differentiation. In Table 11 the molecular weights of the Icelandic MVV type strain (K1514) (Vigne et al. 1982) are compared with that of SA OMVV I strain shown in Plate 21a. No significant difference between the two MVV strains could be detected except for the p14 which was often absent in the South African strain and when present, only faintly so.

Plate 2la Polypeptide pattern of SA - OMV I. The electrophoretic conditions were as described in the legend to Plate 2. Lane S, represents SA - OMV I polypeptides and lane M, low molecular weight markers (Pharmacia Uppsala Sweden).


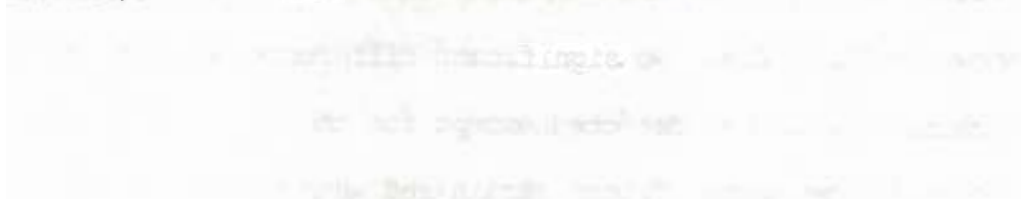


Plate 2lb Comparison of the polypeptide pattern of JSRV and SA - OMV I. The electrophoretic conditions were as described in the legend to plate 2la. Lane J, represents JSRV polypeptides; lane S, SA - OMV I polypeptides and lane M, low molecular weight markers (Pharmacia, Uppsala, Sweden).



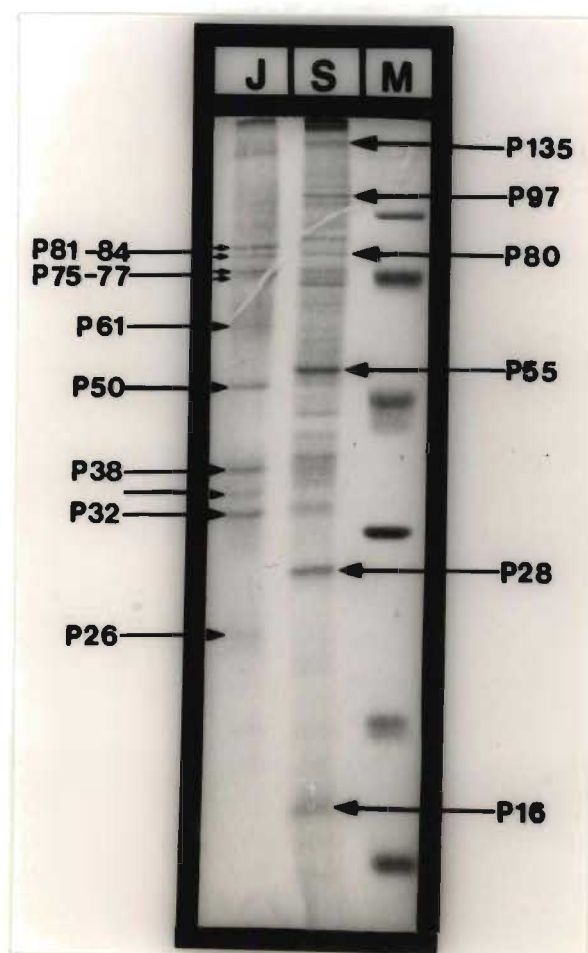
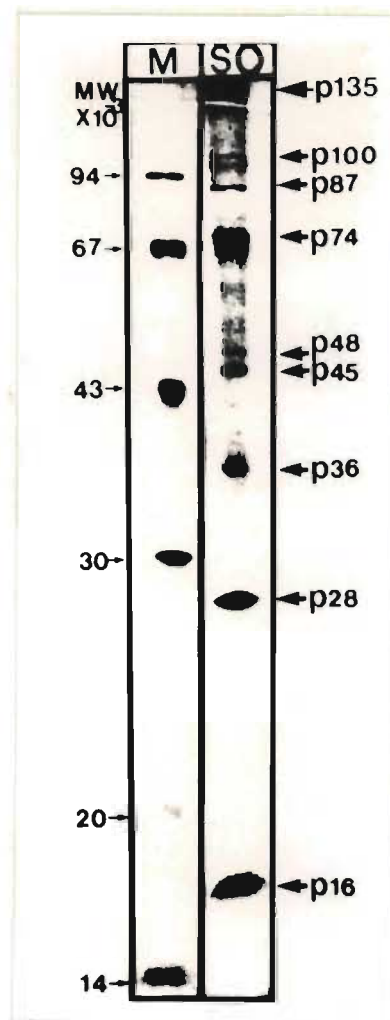


TABLE 11 A comparison of the polypeptide molecular weights of the Icelandic K1514 MVV strain with those of SA - OMVV I, shown in Plate 21A.

SA - OMVV I Mol Wt kd	K1514 (1) Mol Wt kd
135	135
100	97
87	80
48	55
45	50
28	30
16	16
(14)	14

(1) Vigne *et al.*, 1982.

(14) Very faint.

In Plate 21b a polypeptide pattern of SA OMVV I is presented alongside a polypeptide pattern of purified JSRV.

4.2.5 Morphology of SA - OMVV I

The morphology of SA - OMVV I is also identical with that of the Icelandic strain of MVV. A composite picture showing the budding, mature and negatively stained characteristics of MVV is presented in Plate 22. The SA - OMVV I particles observed in the isopycnicallly purified JS/SA - OMVV I lung pellets had smooth outer membranes as shown in Plate 22 (block C). This feature can be used to differentiate between SA - OMVV I and JSRV, as the later virus has surface spikes.

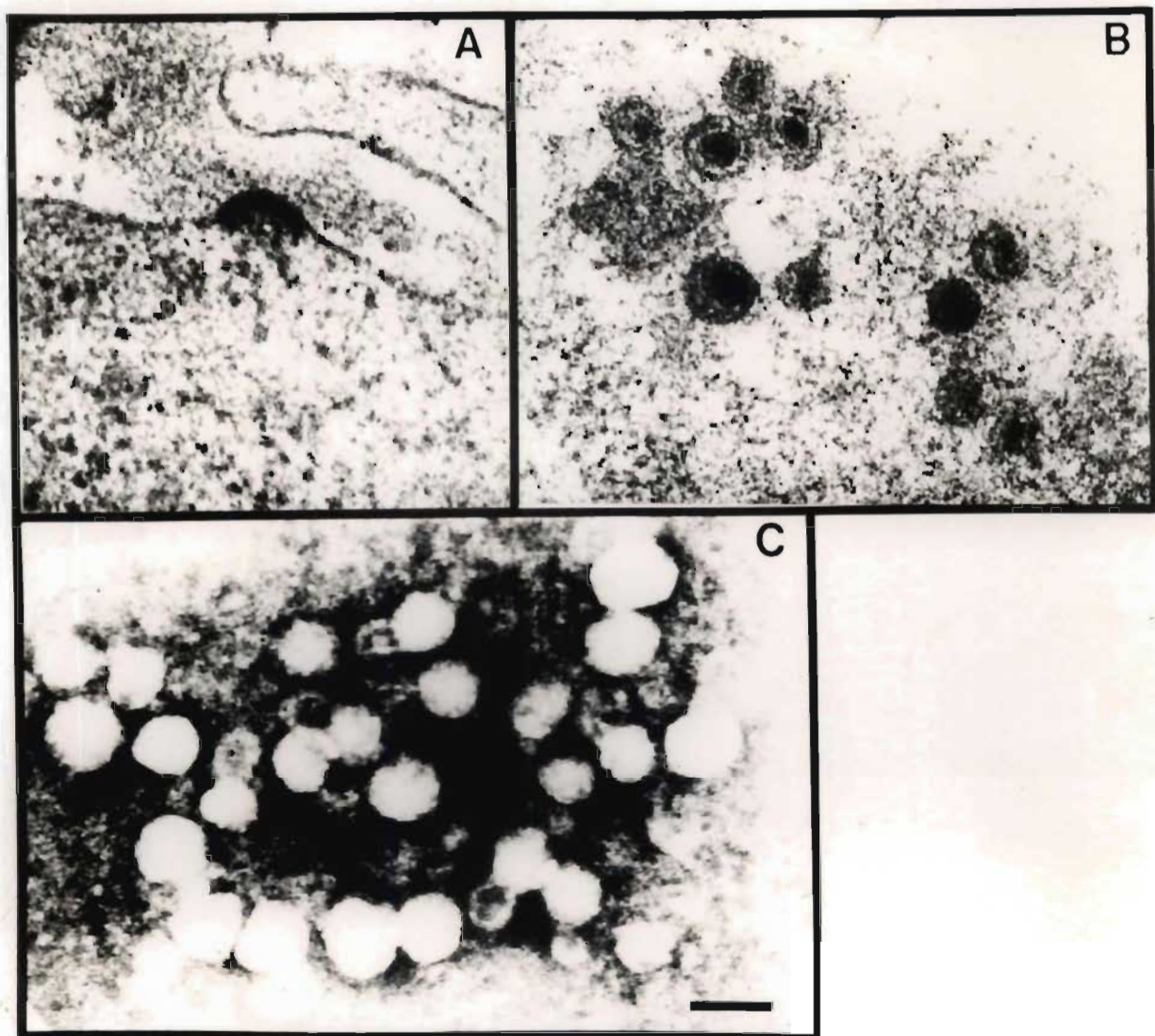


Plate 22 A composite picture showing the morphogenesis of SA OMV
 I. Budding, block A; Mature, block B and negatively
 stained, block C. MVV does not normally have an intra-
 cytoplasmic form. This plate was kindly supplied by Dr.
 A - Payne (Payne et al., 1986). Bar represents 100 nm.

4.3 DISCUSSION

The precise role of SA - OMV I in jaagsiekte is uncertain. However, the high incidence of MVV in JS infected sheep suggest that it is more than mere coincidence.

In this chapter evidence was first presented which shows that SA - OMV I is present in lung washes from sheep diagnosed to be infected with JS. A colleague has also shown, using a direct immunoperoxidase technique on frozen JS lung sections, that both viruses were present in the same lung sections (Williamson, unpublished results). This was most disturbing especially in view of the fact that all the early JSRV purification research was monitored using the RDP assay which cannot distinguish between the two retroviruses. It did explain some conflicting early results, however. In the course of early experiments the presence of more than one RDP peak often led to speculation that cores or virus attached to membrane particles could possibly explain the different density peaks. Especially confusing were results obtained when the gradients were monitored with both the RDP assay and the immunoblot using MPMV as antiserum to detect JSRV and the RDP and blot results would not correlate. Fortunately, their peak densities differ viz. JSRV 1,186 g/ml and SA - OMV I 1,155 and 1,167 g/ml, when isopycnicly purified from JSRV lung wash material (see Fig. 5). With the availability of methods to distinguish between JSRV and SA - OMV I, gradient conditions have been established to optimize purification of JSRV from SA - OMV I (see Fig. 7).

The strength of the immunoblot technique in JS research is that the material to be tested does not have to be pure. If one considers how many proteins are present in lung wash pellets and it is still possible, using an immunoblot technique, to determine whether a JS lung wash pellet is also contaminated with SA - OMV I, its value is readily apparent. Attempts to resolve these problems by direct protein staining would have been futile.

In hindsight it is fortunate that all lung rinse pellets were treated with freon before they were injected into the experimental lambs. This treatment must have reduced the lentivirus concentration considerably. In Fig. 5a and 5b it is shown how the freon removes the lentivirus from the 1,155 g/ml region. This is the density of virus cultured in vitro (see Fig 6). It is not certain why the slightly higher density lentivirus is protected from the freon treatment. It may represent aggregates of virus or cores with membranes or antibodies, protecting it against the organic solvent, but this remains speculative at this stage. It is interesting to point out that SA - OMV was recovered from in vitro cultures that had been infected with non - Freon treated JS lung wash material. Earlier attempts to culture MVV using a similar culture system but infected with freon treated JS lung pellet material, did not result in the recovery of MVV. Although it has not been excluded that those earlier cultures might have been free of MVV and that this might therefore explain the lack of MVV recovery, the evidence suggests that freon treatment prevents MVV infectivity. If this is true then it implies that the 1,168 g/ml SA - OMV I is not infectious supporting the hypothesis of

protection by antibody.

SA - OMV I has a distinct polypeptide pattern that differs from that of JSRV (Plate 22b). Two readily identifiable markers are the p28 and p16 of SA - OMV I. Although JSRV has the p29 which is detected on the immunoblot with MPMV antiserum, this protein is only very faintly detected with Coomassie staining. It is interesting to note in the blot result (Plate 18) that the JSRV p29 and SA - OMV I p28 have the same electrophoretic mobility. Although it is also shown in this blot that they are not serologically related one could speculate that the JSRV might be a mutant lentivirus with a core protein that reacts with antiserum to core polypeptides of type D retroviruses and MVV. DeMartini et al., (1987) also speculated on this possibility but they concluded that, because of the genetic disparity between the lentiviruses and the type D oncoviruses, this was unlikely. A more likely explanation is that the MVV p28 is a distinct protein coincidentally comigrating with the MPMV reactive JSRV band.

The polypeptide pattern of SA - OMV I is nearly identical to that of the Icelandic type MVV strain. The only noticeable difference is the p14 which is nearly always absent in the SA - OMV I strain (Plate 21a). Unfortunately the Icelandic virus is not available in South Africa so that the two viruses could be run alongside each other and a direct comparison made. Nevertheless, a comparison was made in France where the Icelandic MVV strain was co-electrophoresed with SA - OMV II strain. SA - OMV II strain is a French

MVV isolate from the South African 15.4 JS tumour cell culture (Querat et al., 1987). They showed that the four major viral proteins i.e. gp 135, p30, p16 and p14 of SA - OMV II were similar in size to those of the Icelandic strain. Interestingly the p14 of SA - OMV II was also of a very low intensity compared with the p30 and p16. The similarity between these proteins was confirmed serologically using radio - immoprecipitation techniques (Querat et al., 1987).

Morphologically SA - OMV I is also distinct from JSRV. Electron microscopic observations revealed that the South African isolate of MVV buds with a crescent shaped core with no intermediate space (Plate 22) and has immature particles that have an electron dense layer immediately below the viral envelope. Mature particles were membrane bound with an electron lucent perinucleoidal space and round or conical shaped cores. One of the differences between JSRV and SA - OMV (and the other lentivirus members for that matter) is the absence of surface spikes on the MVV particles (Plate 22, block C). The spikes are easily observed in negatively stained JSRV particles and it is this feature that facilitates the distinction between the two viruses when negatively stained samples of gradient purified JS lung rinse pellets are studied (see Table 10).

Since the discovery of the AIDS (HIV I) virus which is also a retrovirus and a member of the family Lentiviridae (Popovic, Sargadharan, Read and Gallo, 1984), there has been much speculation on the immunosuppressive role of MVV. This role could explain

the reason for the co - existence of MVV and JSRV in JS infected sheep lungs. From experiments done in this laboratory to investigate immunosuppression in MVV infected JS and JS free sheep, it was concluded that both viruses (JSRV and MVV) have an immunosuppressive effect, predisposing the host to infection by the other (Verwoerd, Williamson, York, Myer and Huchzermeyer, 1987).

Another explanation for the presence of MVV in JS infected sheep could be that sheep affected with JS have an increased susceptibility to lentivirus infection as there are large numbers of alveolar macrophages in JS lungs (Dawson, Venables and Jenkins, 1985). In agreement with this is the demonstration that alveolar macrophages are the preferential target cell in MVV infected sheep (Gendleman, Narayan, Molineaux, Clement and Ghotbi, 1985).

Whatever the role of MVV in JS, the evidence against a primary role for MVV in causing JS includes the lack of antigenic cross reaction of MPMV (p27) with MVV (p28), lack of MVV RNA in JSRV (Perk and Yaniv, 1977), the failure to transmit JS with SA - OMV I in seventeen sheep (Payne et al., 1986) and finally lack of evidence of MVV infection in individual animals with JS or on certain farms that have a high incidences of JS but are free of MVV.

The involvement of other retroviruses in the JS disease complex is constantly being reassessed. Recently another retrovirus was isolated from bovine and its relationship to JSRV investigated. This virus will be discussed in the following chapter.

CHAPTER 5

THE BOVINE RETROVIRUS

5.1 INTRODUCTION

In 1986, Dr Barnard (Virology section, Veterinary Research Institute, Onderstepoort) observed a number of cells which showed transformed characteristics viz. rounding off, loss of contact inhibition and very rapid cell division, in a calf foetal thyroid (CFTH) cell culture. This culture had been inoculated 42 days previously with white blood cells from a bovine suffering from the sheep associated form of Malignant Catarrhal Fever Virus (MCFV). Further studies of these cells revealed retrovirus like particles under TEM. To exclude the possibility that these particles were Bovine Leukemia Virus (BLV), the only known bovine oncovirus, six sheep were injected with these cells to observe whether any BLV specific antibodies were produced. No serum conversion was observed in any of the sheep injected. However, one of the sheep developed jaagsiekte.

The possibility existed that this virus may have caused that one sheep to develop JS. For this reason the virus was handed over to our department for further investigations. The major objectives of the investigations were to study its biochemical, immunological and morphological characteristics and to compare them with those of JSRV. The approach and techniques developed in studying the JSRV (Chapter 3) and SA - OMV I (Chapter 4) were also applied to this virus. In addition its in vitro and in vivo infectivity, was investigated to elucidate its role, if any, in the etiology of

jaagsiekte.

For clarity this virus will be referred to as Bovine Retrovirus (BRV). "Bovine" from the origin of the virus and "retrovirus" from its reverse transcriptase activity and oncovirus like morphology.

5.2 RESULTS

5.2.1 RDP-activity

In the general introduction it was mentioned that a characteristic of all retroviruses is that they have an RNA dependant DNA polymerase enzyme (RDP). Depending on the retrovirus, the RDP enzyme has a characteristic cation requirement for either magnesium or manganese to function effectively. After demonstrating the existence of RDP - activity, its cation preference was investigated. Serial dilutions of the virus were added to an RDP assay mix containing either magnesium or manganese (see Methods 2.2.1). A control series was also done in parallel for each cation without adding template - primer, to exclude false positive results caused by cellular DNA polymerases.

TABLE 12 RDP activity in BRV pellets using magnesium and manganese as the divalent cation. Parallel samples were tested with and without template - primer (+T/-T).

BRV Dilution	Mg + T	Mg - T	Mn + T	Mn - T
	cpm	cpm	cpm	cpm
1;1	328	284	8 312	984
1:2	144	130	4 696	1 030
1:4	86	84	2 962	686

T = template primer (poly rA oligo dT¹⁰, Boehringer Mannheim)

It is clearly shown that BRV particles have RDP activity which has a preference for manganese as the divalent cation requirement (Table 12). There was, however, a low but significant cDNA synthesis when no exogenous template was included in the assay. This

could be due to endogenous DNA polymerase activity or to the RDP utilizing some endogenous template.

The RDP activity of BRV was also compared with that of JSRV and SA - OMV I using the same assay system and both divalent cations. The results of this comparison are presented in Table 13.

TABLE 13 A comparison of the RDP - activity present in BRV, JSRV and SA - OMV I showing the cation preference for each virus.

Virus	2+ Mg	2+ Mn
	cpm/20 ul	cpm/20 ul
BRV	540	51 608
JSRV	4 292	548
SA-OMV I	56 042	4 978

It is clearly shown that both JSRV and SA - OMV I have a preference for magnesium whereas BRV has a definite preference for manganese.

5.2.2 Immunoblot

Having established the presence of reverse transcriptase (RDP) activity, a sample of the BRV was fractionated on a 12,5% SDS-PAGE and transferred to nitrocellulose (see Methods 2.8.1). Using an immune overlay technique (see Methods 2.8.2) and serum against the group specific antigen of MPMV, this now being the standard approach to confirm the presence of JSRV, it was shown that the BRV pellet contained a protein that strongly cross-reacted with this serum (see Plate 23). This protein had an estimated molecular

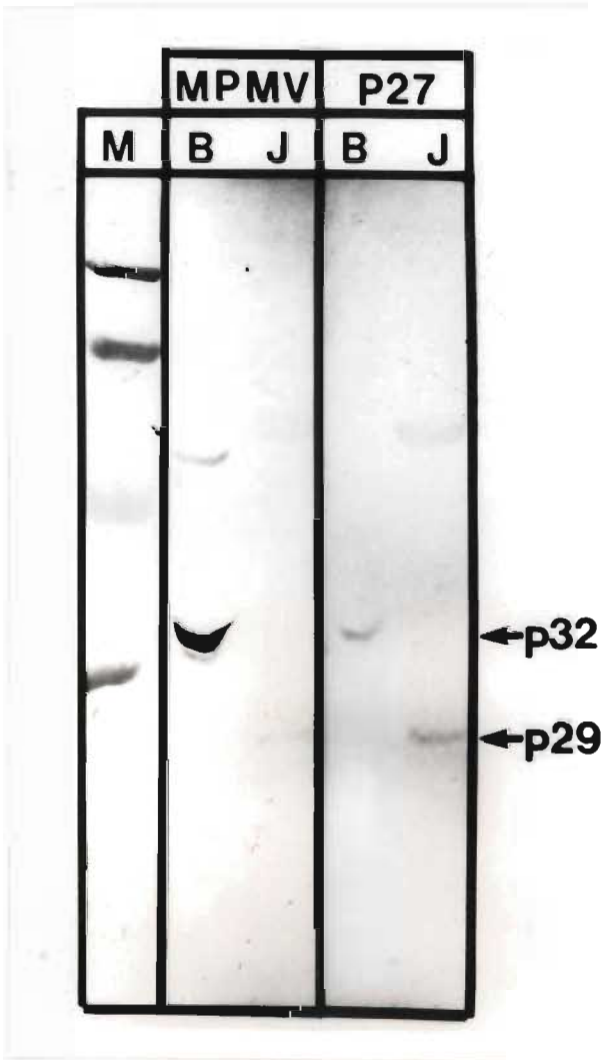
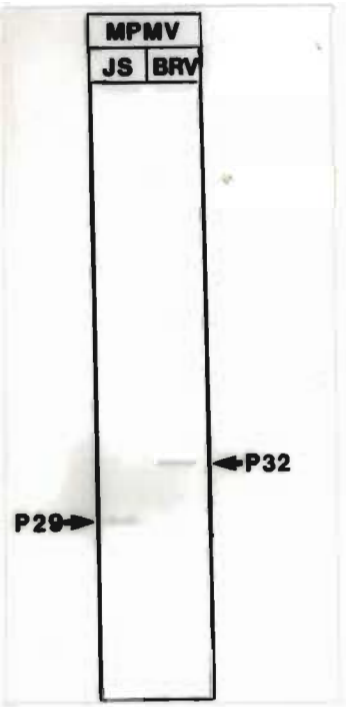
weight of 32 000 dalton and was, therefore not the same size as the p29 protein of JSRV.

To confirm that this cross - reaction was due to antibodies directed against the p27 of MPMV, a very simple yet effective technique was performed. MPMV antigen was separated on a preparative gel and transferred to nitrocellulose. The whole strip was then overlayed with MPMV antiserum, diluted 1:200, and incubated overnight. After washing (see Methods 2.8.2.2) a strip was cut off each side of the nitrocellulose and incubated in peroxidase conjugate. The central portion was left in buffered saline. The position of the p27 band was identified 1 hour later when peroxidase substrate was added to the two side strips (see Methods 2.8.2.2). After developing, the two side strips were placed alongside the central portion of nitrocellulose and the p27 band cut out. This strip, which now contained antibodies bound to p27, was soaked in 10 ml 0,02 M glycine/ HCl buffer (pH 2,9) for 10 min to release the antibodies. The strip was removed and 500 μ l 2M Trizma base added to neutralize the acid. These antibodies were then used in an immunoblot technique and shown to react with the p32 of BRV (see Plate 24).

To confirm that BRV did not cross react serologically with the lentivirus (SA - OMV V I) or Bovine Leukemia virus (BLV), an immunoblot was performed including the relevant controls. It is evident in Plate 25 that there is no reaction between BRV and sera against MVV and BLV.

Plate 23 An immunoblot showing the polypeptides of JSRV (lane JS) and BRV which react with MPMV (p27) antiserum (see Methods 2.8.2.2). Antiserum was used at 1:400 dilution.

Plate 24 An immunoblot result showing the reaction of MPMV antiserum and anti - MPMV p27 serum prepared as described in the above text, with BRV lane B and JSRV lane J.



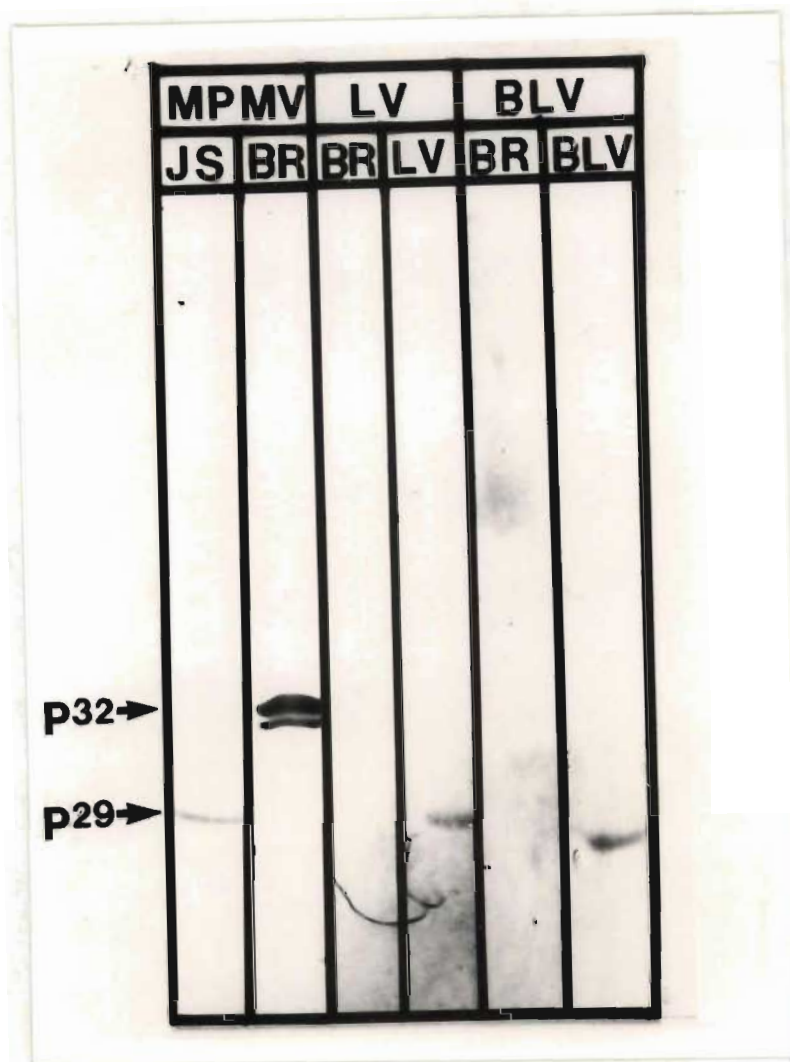


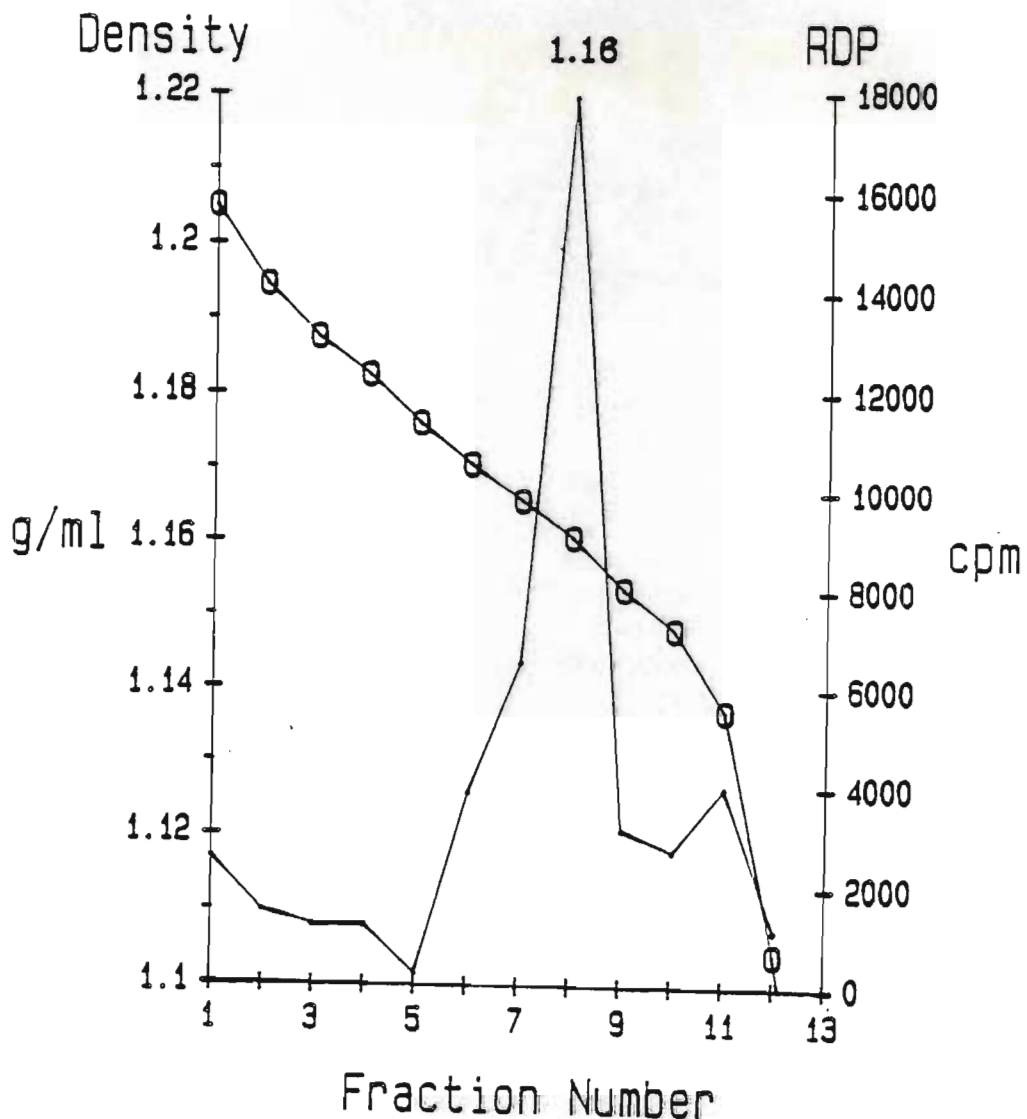
Plate 25 An immunoblot comparing the reaction of BRV with sera against MPMV, MVV (heading LV) and BLV. MPMV anti-serum was used at 1:400 and MVV and BLV at 1:50. Note the absence of any reaction between BRV and both MVV and BLV antisera.



Fig 8. Isopycnic centrifugation of BRV pellet. Virus (1,5 ml) was layered on a 25 - 45 % sucrose gradient in a Beckman SW 41 tube. Centrifugation was overnight (80 000 x g, 4 C). Density (○) and RDP activity (●) were measured in selected fractions. The RDP activity peaked at fraction 7 which has a density of 1,16 g/ml. Selected fractions were run on SDS-PAGE and immunoblotted (see Plate 26). The p32 protein band is most intense in fractions 6, 7, 8 and 9 centering around a density of 1,16 g/ml.

5.2.3 RDP and immunoblot monitoring of gradient purified BRV

Although the pellets containing BRV were shown to contain RDP activity and a protein that cross - reacted with serum against the group specific antigen of MPMV, it still had to be ascertained whether these two characteristics were from the same particle. To answer this question the pelleted material was layered onto a sucrose gradient and centrifuged to isopycnic equilibrium. Fractions of the gradient were then monitored for RDP activity and the presence of the 32 000 dalton protein (p32). It is clear in Figure 8 that the RDP peak and the p32 peak (Plate 24) coincide at fraction 8, which has a density of 1,16 g/ml.



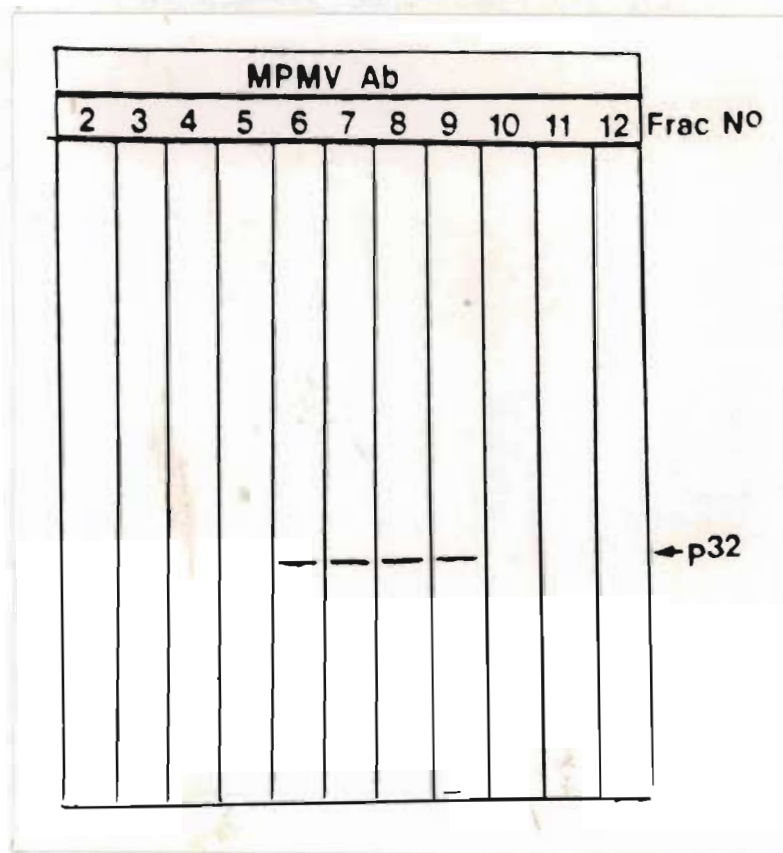


Plate 26 An immunoblot result showing the reaction between BRV fractions taken from Figure 8 and MPMV antisera. Note the p32 at fractions 6, 7, 8 and 9. Antiserum was used at 1:400 and immunoblot conditions were as described in Methods 2.8.2.

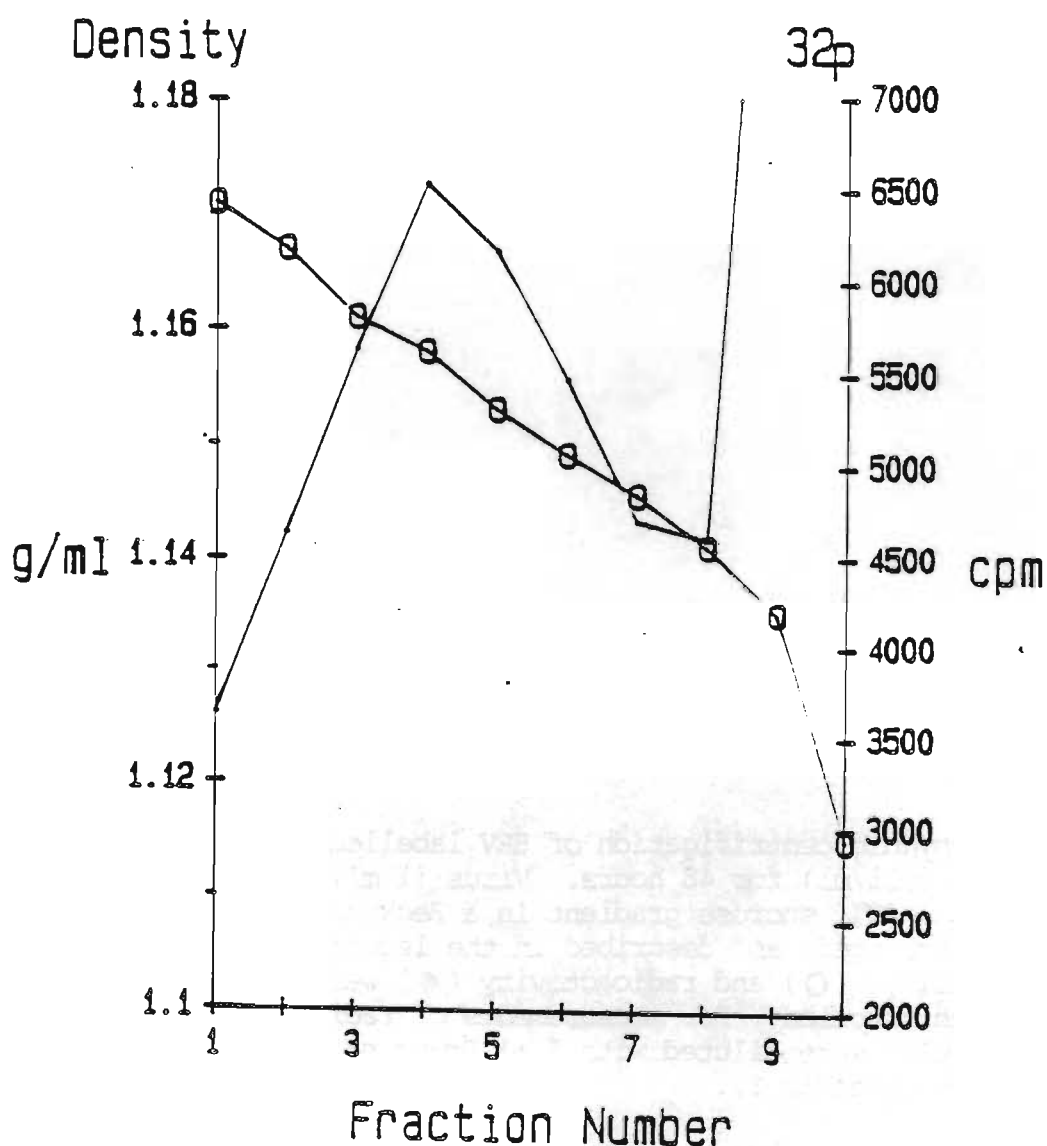
Plate 50 of manuscript showing the results of isopycnic centrifugation of BRV labelled with ^{32}P -orthophosphate. The figure shows a sucrose gradient with density (O) and radioactivity (●) plotted against fraction number. A prominent radioactivity peak is visible between fractions 5 and 7, corresponding to a density of approximately 1.16 g/ml.

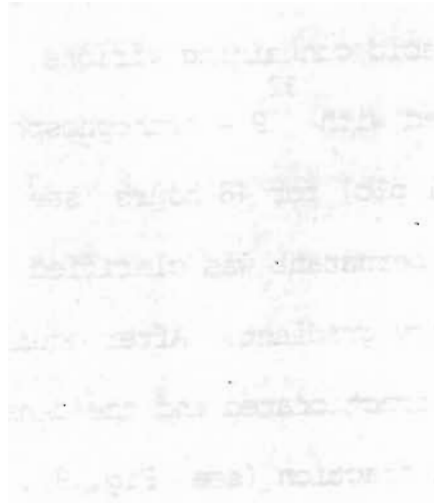
32

Fig. 9 Isopycnic centrifugation of BRV labelled with ^{32}P -orthophosphate (2,5 $\mu\text{Ci/ml}$) for 48 hours. Virus (1ml) was layered on a 25 - 40 % sucrose (w/v) gradient in a Beckman Ti SW 41 tube. Centrifugation was overnight (80 000 x g, 16 h, 4 C). Density (O) and radioactivity (●) were determined for each fraction. For radioactive measurements 50 μl of sample was diluted in 5 ml Brays scintillation fluid (see Appendix I). Note the radioactivity peak between fractions 5 and 7 around 1,16 g/ml.

5.2.4 Genetic material associated with BRV

To determine whether the 1,16 g/ml density particle consisted of complete nucleic acid containing virions, confluent transformed cells were labelled with ^{32}P - orthophosphate (2,5 uCi/ml, Amersham International plc) for 48 hours (see Methods 2.3.3.1). The labelled culture supernatant was clarified, pelleted and layered on a sucrose density gradient. After equilibrium centrifugation the gradient was fractionated and the density and radioactivity determined for each fraction (see Fig. 9).

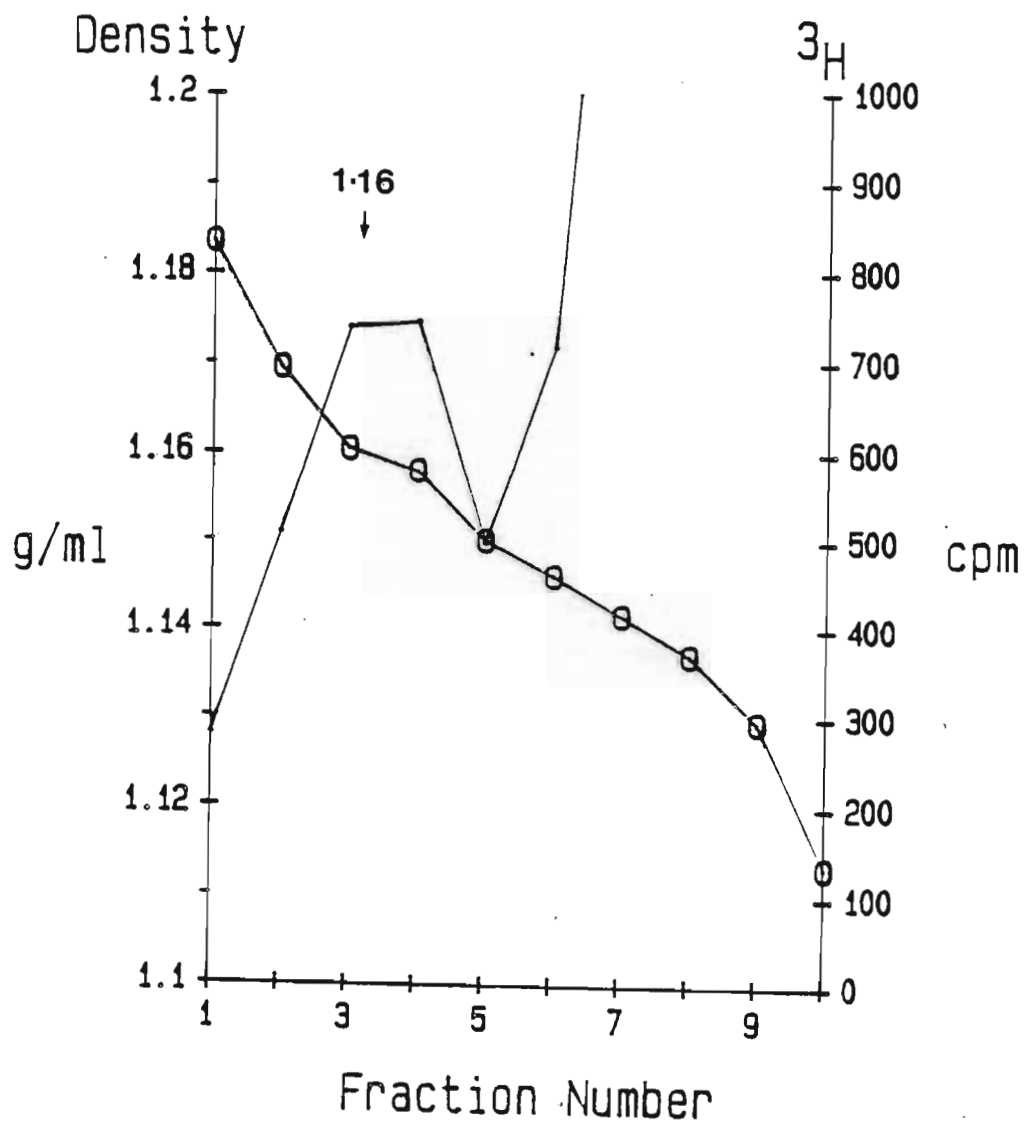




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Fig. 10 Isopycnic centrifugation of BRV labelled with ^3H -Uridine (70 $\mu\text{Ci/ml}$) for 48 hours. Virus (1 ml) was layered on a 25 - 40% sucrose gradient in a Beckman SW 41 tube and centrifuged as described in the legend to Figure 11. Density (O) and radioactivity (●) were determined for each fraction. For measurements of radioactivity 100 μl samples were diluted with 5 ml Brays scintillation fluid (see Appendix I).

Although a peak of activity is seen at 1.16 g/ml, the concentration of the virus was not high enough to detect the ^{32}P using the immunoblot technique. Due to its high specific activity, detection of ^{32}P is very sensitive and relatively cheap, however, it labels both RNA and DNA as well as phosphoproteins. To determine whether the genetic material was RNA, ^3H - Uridine (70 uCi/ml) (Amersham International plc.) was added to a BRV producing culture and an equivalently aged normal CFTH culture for 48 hours, as control. The clarified culture supernatants were pelleted then layered onto sucrose gradients and centrifuged to isopycnic equilibrium (see Methods 2.5.1.1). The fractions were then monitored for density and radioactivity (see Fig. 10).



In Figure 10, a radioactive peak is seen at the 1,16 g/ml region when ^3H - uridine was used, confirming that the particle has RNA as its genetic material. No label was found when ^3H - dTTP was used to label DNA.

5.2.5 BRV polypeptides

BRV was purified by recovering the 1,16 g/ml region of an isopycnic density gradient and its proteins separated on a 12,5 % SDS - Polyacrylamide gel so that its polypeptide pattern could be determined (Plate 27). The polypeptide pattern is very interesting in that it consists of 7 major proteins viz. p122; p84; p74; p45; p32; (p30) and P16. As far as concentration is concerned, p32 is the major component followed by p16. P32 and p30 are often observed as a doublet band and because they are so close electrophoretically, p30 is often not seen.

5.2.6 BRV production studies

In order to study the release of BRV from the transformed cells, culture supernatants from both CFTH and BRV transformed cells were monitored over a period of 16 days for the presence and intensity of p32, using an immunoblot technique and MPMV (p27) antisera. The results are tabulated in table 14.

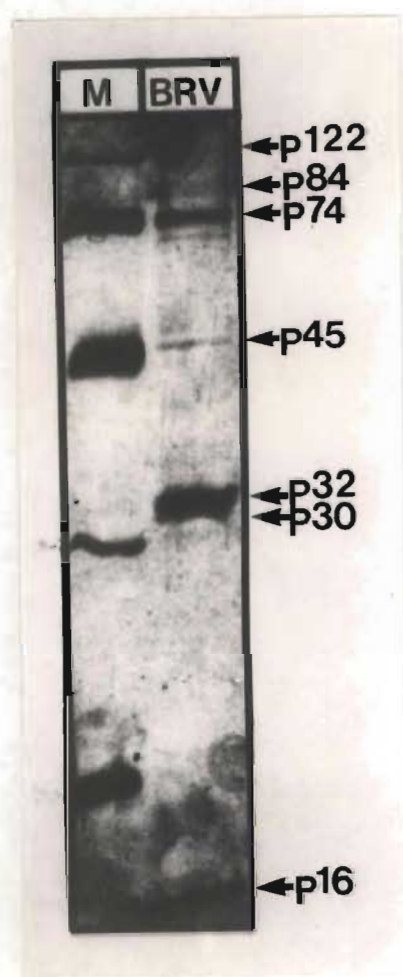


Plate 27 Polypeptide pattern of purified BRV (lane B). Electrophoresis was carried out in a 12, 5 % SDS polyacrylamide gel (see Methods 2.8.1). Lane M represents low molecular weight markers. The molecular weights of the BRV polypeptides are indicated on the right.

TABLE 14 The results obtained when CFTH and BRV transformed cells were monitored, at different days after seeding, for the production of p32, as a measure of BRV production.

Days after seeding	p32	
	BRV	CFTH
1	-	-
2	-	-
4	+	-
7	+++	-
9	++	-
11	++	-
14	++++	-
16	+++	-

+, represents the intensity of p32 from the immunoblot results. Four +++, is the intensity of the positive control and one +, is approximately 25 % of the positive control. (-), indicates the absence of p32.

From the results it is apparent that BRV is released into the supernatant after the 4th day, the maximum production occurring at days 7 and 14.

5.2.7 Immunocytochemical studies of BRV

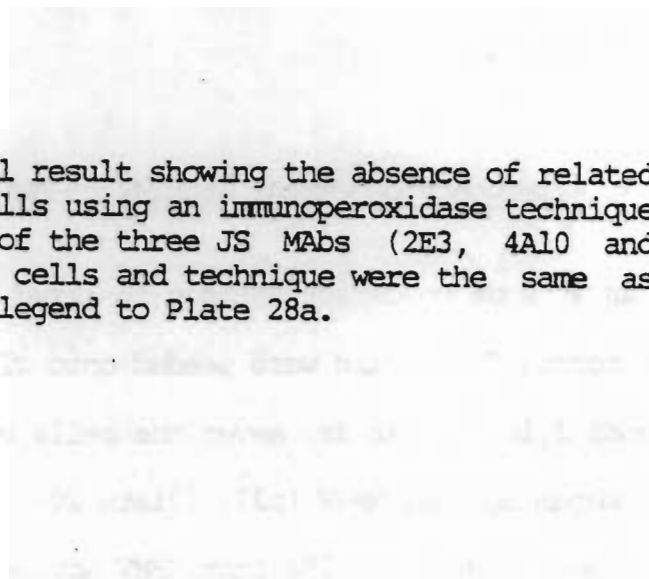
In an attempt to detect antigen in fixed cells, BRV transformed and normal CFTH cells were seeded onto glass chamber slides (see Methods 2.10.3). At day seven the cells were fixed and overlayed with serum against MPMV (p27) (Plate 28a) (see Methods 2.10.4). It is clear from Plate 28a that MPMV antiserum detects antigen in nearly all transformed cells. No antigen was produced in CFTH cultures of the same age. When BRV - transformed cells were overlayed with MVV antiserum no related antigen was detected (Plate 28b). This is in agreement with the blot results where anti- MVV serum failed to detect related antigen in BRV (see Plate 25).

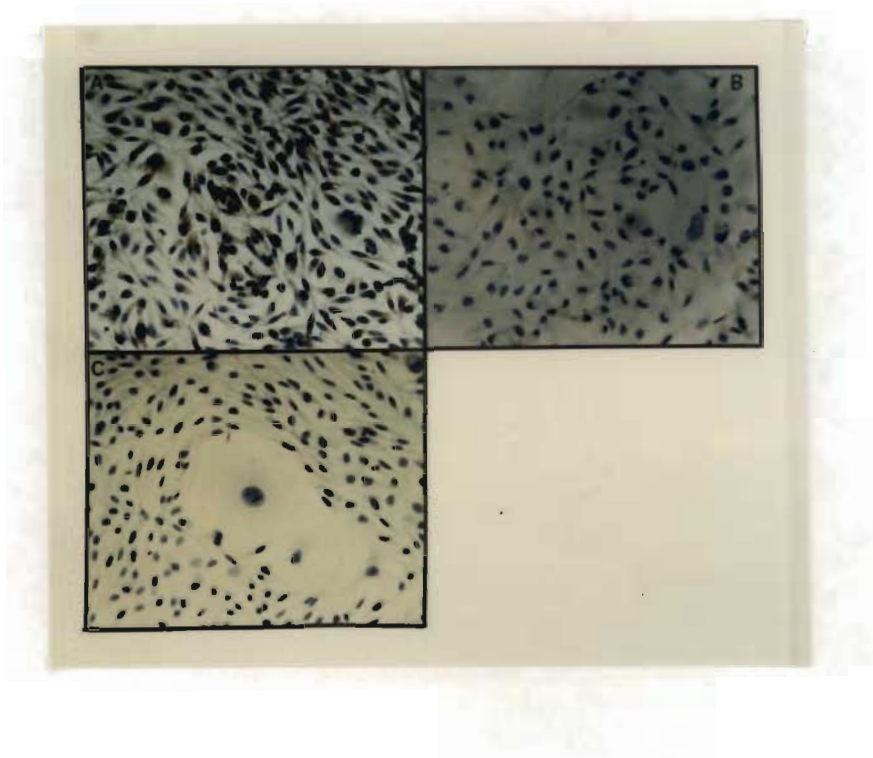
Plate 28a Immunocytochemical results showing the antigen (brown staining)→detected with MPMV(p27) antiserum and an indirect immunoperoxidase technique (see 2.10). BRV transformed cells were seven days old. Note the high percentage of cells that stain brown.

- b Immunocytochemical result showing that there is no antigen detected in BRV transformed cells with MVV antiserum. The technique used was as described in the legend to Plate 28a.



- c Immunocytochemical result showing the absence of related antigen in BRV cells using an immunoperoxidase technique and a cocktail of the three JS MAbs (2E3, 4A10 and 11G11). Age of cells and technique were the same as described in the legend to Plate 28a.





As the JS specific monoclonal antibodies 2E3, 4A10 and 11G11 could not be used in an immunoblot technique, they were tested in an immunocytochemical technique for any serological cross reaction with BRV. However, as seen in Plate 28c, no related antigens were detected by the MAbs.

5.2.8 Morphology of BRV

Transmission electron microscopic observations of negatively stained material covering all the fractions when BRV was isopycnically purified, revealed very clean virus like particles in the 1.16 g/ml region. These particles morphologically resembled the members of the family Retroviridae (Plate 29f and g).

Intracytoplasmic type A particles were present in positively stained cell culture preparations (Plate 29a). Their morphology was typical of that seen in type D retroviruses (Todaro, Benveniste, Sherr, Schlom, Schidlovsky and Stephenson, 1978). The inner shell of the core particle was more electron dense than the outer shell (see Plate 29a). Particles were observed budding from the cell membrane with both complete (Plate 29c) and incomplete cores (Plate 29b). (Incomplete cores are a characteristic of type D retrovirus budding (Todaro et al., 1978). Immature extracytoplasmic particles were commonly seen with a relatively electron lucent perinucleoidal space (Plate 29d). The mature extracellular particles were morphologically distinct from the primate type D retroviruses. They were electron dense and resembled the JSRV particles (Plate 29e).

Plate 29a Positively stained BRV showing intracytoplasmic type A particles (\Rightarrow). Note the inner shell of the core is more electron dense than the outer shell.

b Positively stained BRV infected cells showing particles budding with incomplete cores.

c Positively stained BRV infected cells showing particles budding with complete cores.

d Positively stained BRV infected cells showing mature extracellular particles (\Rightarrow). Note the clear central core and relatively electron lucent perinuclear space.

e Positively stained BRV infected cells showing mature extracellular particles. Note the electron dense central portion of the particles.

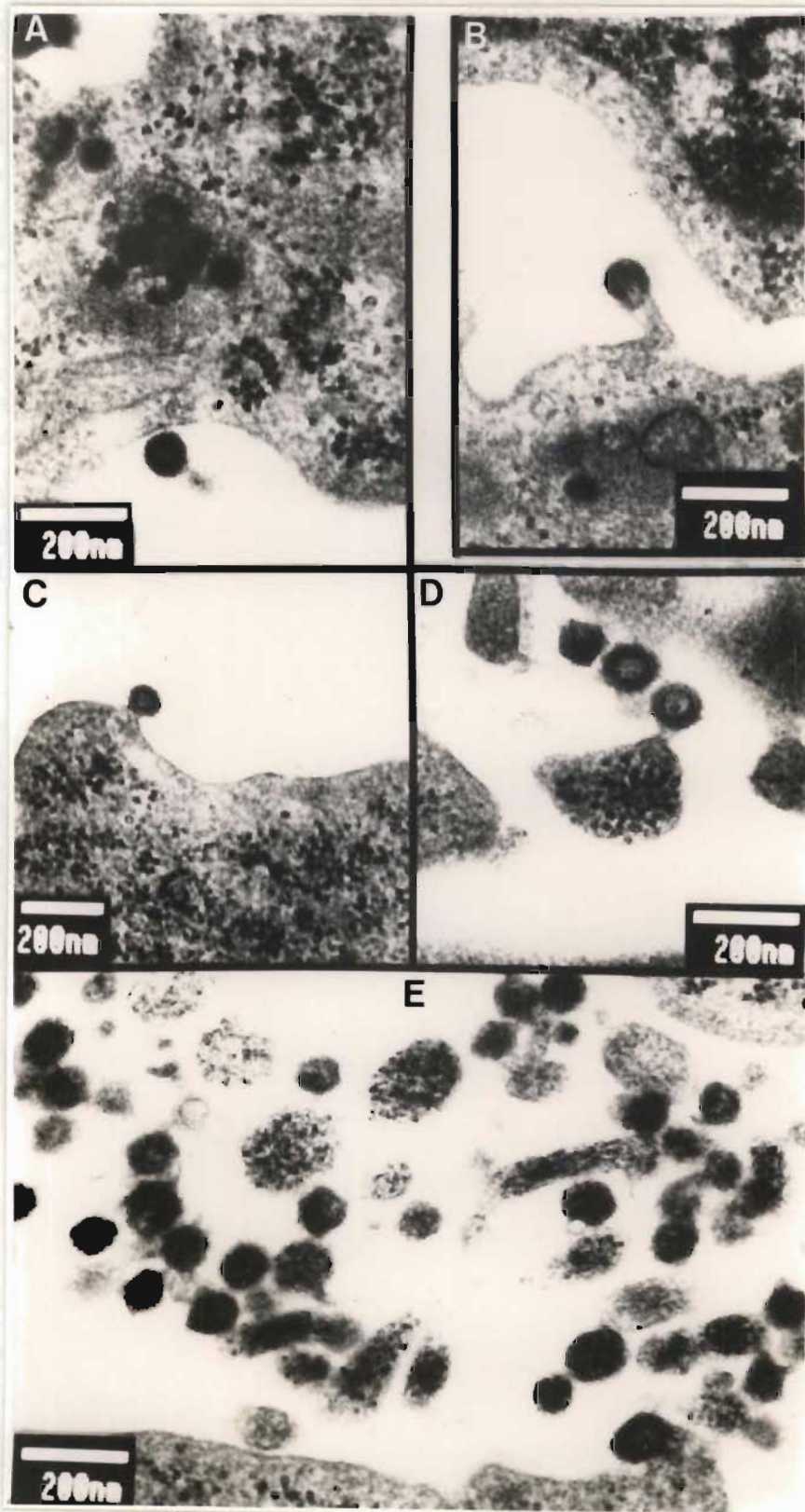
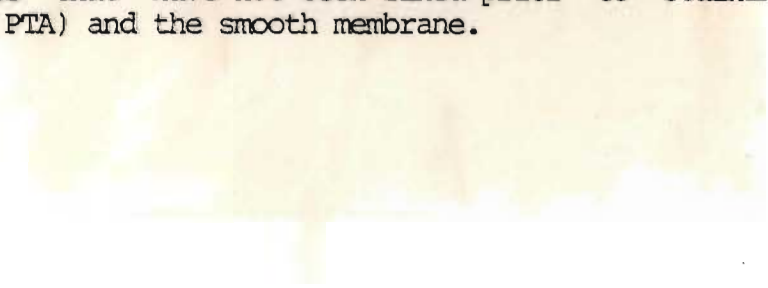

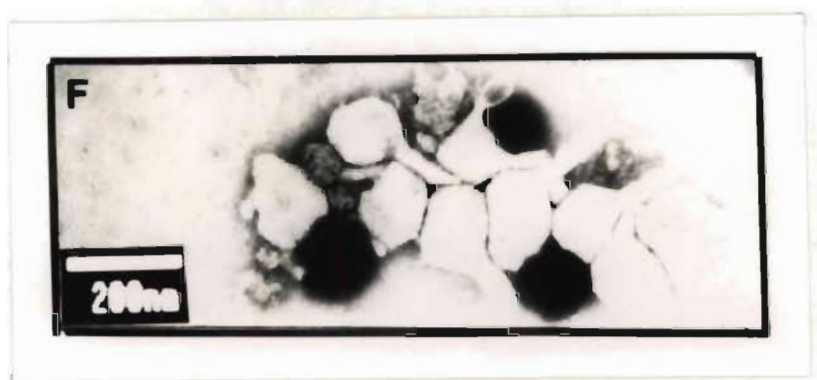


Plate 29f PTA negatively stained BRV particles (see Methods 2.17) from the 1,16 g/ml density region (see Fig 8). Note the tails (this is a common observation of retroviruses that have not been fixed prior to staining using PTA) and the smooth membrane.



g Uranyl acetate stained BRV particles (see Methods 2.17) from the 1,16 g/ml density region. Note the centrally located nucleoid and the absence of surface spikes.





In some particles it was possible to distinguish a centrally located nucleoid. However, on negative staining, BRV did not have the surface spikes observed in JSRV particles (Fig 29f). The position of the core could be more clearly observed in uranyl acetate (UA) negatively stained particles (Plate 29g) than phosphotungstic acid (PTA) stained preparations (Plate 29f).

5.2.9 Transformed nature of BRV infected cells

Altered cell morphology, chromosome abnormalities and loss of contact inhibition are major criteria for transformation. In addition, the BRV infected cells were inoculated subcutaneously into four nude mice to confirm their transformed nature. Within three weeks all four nude mice had developed large tumours (Plate 30). Mice injected with normal CFTH cells did not develop tumours.

TEM observations of the cells from the nude mice tumours revealed virus particles identical to the BRV (results not presented). Mr N. Nel, Irene Research Institute examined these cells and confirmed that the chromosome number and pattern are very much like those of transformed cells.

5.2.10 Possibility of HeLa cell contamination

Oncovirus like particles which were shown to cross - react serologically with MPMV, have previously also been isolated from HeLa cells (Watson, Mollung, Gelderblom and Bauer, 1974). Since then there have been numerous reports of MPMV related oncoviruses being isolated from transformed cultures. However, many of these

cultures were later shown to be contaminated with HeLa cells which are notorious for contaminating cell cultures (Bauer, Daams, Watson, Molling, Gelderblom and Schafer, 1974). To exclude the possibility of HeLa cell contamination, a HeLa cell culture was prepared in a separate laboratory, at this Institute. Material from the HeLa cell culture was pelleted and co - electrophoresed alongside a similarly prepared BRV pellet in SDS - PAGE and an immunoblot performed to determine whether the HeLa cells produced the p32. In Plate 31 it is shown that the HeLa cell pellet does not produce the same protein.

In addition to testing for the presence of p32, cells were sent to Mr Neil Nel at Irene Research Institute to be caryotyped. These cells included BRV transformed cells, HeLa cells and CFTH cells. He confirmed that the BRV transformed cells were of bovine origin, the same as CFTH cells, but that they had very high numbers of chromosomes, a characteristic of transformation. The HeLa cells were clearly human and could not be confused with the BRV cells.

5.2.11 Infectivity of BRV

5.2.11.1 In vitro

To investigate the infectivity of BRV in vitro, clarified BRV cell medium (7 day old culture) was freeze thawed twice (to ensure that no viable cells remained in the supernatant) and then added to normal CFTH cultures. In parallel clarified culture supernatants were passed through 0,22 um filters to exclude the possibility of freeze - thawing being responsible for lack of infectivity. In

both cases no transformation of the CFTH cells was observed.

5.2.11.2 In vivo

The infectivity experiments where BRV infected cells/virus were injected into sheep were repeated in our department. In this experiment 4 lambs were injected intratracheally with 4 ml transformed cells (1×10^7 cells /ml) and intravenously with 0,5 ml pelleted culture medium, from 7 day old cultures. One year later, when one of the sheep started showing advanced JS symptoms, all four sheep were bled and their sera tested for antibodies to BRV p32 using an immunoblot technique. The results of this immune blot have been summarized in Table 15. For clarity these sheep are numbered 1; 2; 3 and 4. Referring to the immune blot result it is important to note that sheep number 2 gave a strong positive reaction to the p32 protein whereas sheep numbers 1 and 4, showed a faintly positive reaction. Sheep number 3, on the other hand, was negative.

Table 15 Diagnosis and characteristics of four sheep injected with BRV and slaughtered one year later.

Sheep No.	JS symptoms before slaughter	JS diagnosis after slaughter	p32 antibodies (blot result)
1	- VE	++	faint
2	- VE	- VE	++
3	advanced	+++	- VE
4	- VE	+	faint

+'s are used to indicate the degree of JS infection (lane diagnosis) and degree of intensity (heading p32 antibodies). One + is low and +++ is high.



Plate 30 A nude mouse three weeks after it was subcutaneously inoculated with 5×10^6 BRV infected cells. Note the resulting tumour, confirming the transformed nature of the cells.

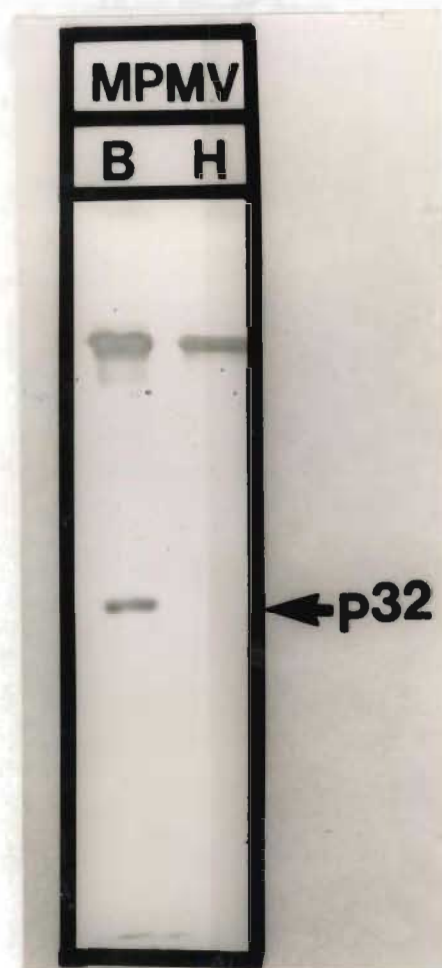


Plate 31 An immunoblot result showing the absence of BRV p32 in HeLa cell culture medium prepared in the same way as BRV. Heading MPMV, represents the antiserum used at 1:400 dilution (see Methods 2.8.2).

When the immune blot results and the post mortem diagnoses are considered together (Table 15) it is interesting to note that sheep number 3, which had the most advanced JS symptoms, had no antibodies to p32 whereas sheep number 2, which was diagnosed to be JS - VE, had the strongest antibody titre. Sheep number 3 was housed together with sheep 4 and might have infected it. However, sheep 1 and 2 were housed in a separate room from sheep 3 and 4.

On purification of the lung wash pellet from sheep number 3 it was shown that RDP activity and JSRV p29 antigen peaked at a density of 1,186 g/ml, confirming the presence of JSRV. No p32 antigen was present in the purified or crude pellet. Interestingly though, there were antibodies in the lung wash (mainly IgA) that were shown to be specific for the p32. The implications and relevance of this finding have to be further investigated.

5.3 DISCUSSION

It was shown in this chapter that the virions isolated from the transformed CFTH cells are members of the family Retroviridae. Evidence supporting this includes biochemical, immunological and morphological characteristics. Considering the biochemical evidence first: Particles were recovered from cell free culture supernatants and shown to have a buoyant density in sucrose of 1,16 g/ml (Fig 8). These particles had RNA as their genetic material (Fig. 9). For a reason not yet established RNA labelling was relatively inefficient. Both ^3H - uridine and ^{14}C - uridine (the latter results are not shown) were used at concentrations higher than normally recommended for retroviruses, and the cells were labelled for different periods. The low rate of incorporation could be due to a low concentration of RNA in mature BRV particles or to the fact that the labelling conditions were not optimal. It is probably a combination of both. There was also no labelling at ^3H - dTTP when 1,16 g/ml when ^3H - dTTP was used to label DNA.

In Table 12 it is shown that BRV has an RNA dependant DNA polymerase (reverse transcriptase) enzyme that has a preference for manganese as its divalent cation requirement. In addition to the reverse transcriptase activity and labelled RNA being associated with these particles at 1,16 g/ml, the particles were also shown to contain a protein of molecular weight 32 000 dalton. This protein cross-reacts with serum against the group specific antigen of MPMV, p27 (Plate 23). As shown in chapters 3 and 4, this serum also cross reacts with a 29 000 dalton protein of JSRV.

Antibodies eluted from the MPMV p27 band cross reacted with the p32 and p29 of BRV and JSRV, respectively. Thus the result presented in Plate 24, confirms that both the BRV p32 and JSRV p29 are serologically related to MPMV p27.

Serologically BRV is not related to either SA - OMV or BLV (see Plate 25), the latter being a common bovine retrovirus. In the general introduction it was mentioned that a retrovirus was isolated from an American Bison in very much the same way as Dr Barnard isolated BRV (Amborski et al., 1987). The Americans did not determine whether their virus was serologically related to MPMV or not. However, they did test their virus with serum against Bovine Syncytial Virus (BSV), Bovine Maedi - like Virus (BMV) and Bovine Leukemia Virus (BLV) and showed that their virus cross - reacted with serum against BSV (using an indirect fluorescent test), but not with BMV and BLV.

Serum against BSV is not as yet available in South Africa, but it will be interesting to determine whether it reacts with BRV when it is received. Bovine syncytial viruses have been detected in normal and compromised cattle affected with lymphosarcoma (Malmquist, Van der Maaten and Boothe, 1969). These viruses are strongly cell associated and cannot be detected if cell - free isolation techniques are used (Amborski et al., 1987). Syncytial viruses are also non - transforming and have not been associated with any disease (Matthews, 1982). Morphologically BSV and BRV are different. BSV has prominent spikes on the surface of budding and extracellular particles (Clarke, Bishop and Mac Ferran, 1970)

whereas spikes are clearly absent from BRV (see Plates 29b and c).

Negatively stained fractions from the 1,16 g/ml region were observed under the TEM and shown to be smooth i.e. they lack surface spikes (see Plate 29g). Although the mature extracellular BRV particles resembled the JSRV particle, the absence of surface spikes also distinguishes these two viruses. A study of the morphogenesis of BRV revealed that there were budding particles with complete (Plate 29c) and incomplete cores (Plate 29b). The latter is a feature of the type C oncoviruses and lentiviruses, although both types of budding have also been observed for MPMV (Todaro et al., 1978). Most of the morphological features of BRV are similar to those of MPMV, except for the mature extracellular particles which are quite different to MPMV, but interestingly, very similar to JSRV. However, the absence of surface spikes differentiate BRV from JSRV.

The polypeptide composition of BRV is quite similar to that of JSRV when the molecular weights are compared:

JSRV: p26; p29; p32; p38; p50; p75 - 77; p81 - 84

BRV: p16; p30; p32; p45; p74; p84; p122

Even so, their polypeptide patterns are still easily distinguished from one other as BRV has a distinctive very intense p32 doublet. When the polypeptide molecular weights of BRV are compared with those of the other retroviruses (see Chapter 6) it is obvious that BRV has a few more high molecular weight proteins than those of the prototypes of B; C and D oncoviruses. In this respect BRV

resembles MVV which has a p14, a p16, a p28 as well as a few higher molecular weight proteins. Nevertheless, it still remains clear that BRV is serologically related to MPMV and JSRV but not to MVV.

Purified cell-free BRV does not appear to be infectious in vitro. This was concluded after cell free preparations of the virus were shown not to transform CFTH cells. It is possible that BRV might be replication defective and that it requires a helper virus for its replication. It is known that many of the oncoviruses that contain oncogenes are defective in one of its other genes and can not replicate in the absence of a suitable helper virus (Lowry, 1985). On the other hand, infectious BRV may be strongly cell associated. The nude mouse experiment confirmed that the BRV - producing cells are transformed and that the tumour formed in the mouse also contained BRV (as observed by TEM). The fact that three out of four sheep injected with BRV developed JS suggests that there were infectious particles in the inoculum. Whether it was cell-free BRV or cell-associated BRV still has to be elucidated. There is also the possibility that BRV could act as a helper virus for a defective JSRV.

According to the immunocytochemical staining when MPMV(p27) anti-serum reacted with BRV infected cells (Plate 28a), nearly every cell was producing related antigen. It is not clear whether this reflects virus or antigen production. Nevertheless, this technique revealed that there is a large amount of p32 being synthesized. MVV antisera (Plate 28b) and a cocktail of the JS specific MAbs

(Plate 28c) did not react with any antigen being produced by these cells using an indirect immunoperoxidase technique.

The origin of BRV is still a mystery. Could it be that a cell in the CFTH culture spontaneously transformed and in so doing activated a suppressed provirus? An example of such a case was that described by Hooks, Gibbs, Chopra, Lewis and Gujdusek (1974). They reported that a human brain culture spontaneously transformed. Further observations of these cells revealed oncovirus like particles under the TEM. The question still remains that if an oncovirus is isolated from a spontaneously transformed culture, is a cellular gene or the virus responsible for transformation? The report by Amborski et al., (1987), where they isolated a retrovirus from bison material affected with MCFV suggests that the origin of BRV may be linked to the MCFV infection. In accord with this is the report that herpes viruses can activate HIV. As MCFV is a herpes virus it is not unlikely that BRV could have been activated in a similar manner.

To summarize the biochemical, immunological and morphological characteristics of BRV: A virus has been isolated that has a density of 1,16 g/ml in sucrose. Particles at this density have RNA as the genetic material and 8 polypeptides of molecular weights ranging from 14 000 to 122 000 dalton. One of the proteins (p32) is serologically related to the group - specific antigen of MPMV and JSRV. The BRV is not serologically related to SA - OMV I nor to BLV. Morphologically BRV resembles the prototype of the type D oncoviruses and JSRV. However, it is different from both

and is possibly a novel member of the family Retroviridae. Considering its association with transformation and its manganese dependant reverse transcriptase enzyme it is probably a member of the type C oncoviruses.

CHAPTER 6

GENERAL DISCUSSION

Three viruses have been isolated that have, in some way, been associated with the JS disease complex. The proof is overwhelming that a retrovirus is involved in the etiology of this disease. This includes an experiment where increasing amounts of reverse transcriptase containing particles resulted in a shorter incubation period before the onset of clinical JS symptoms (Verwoerd and Williamson, 1981). These particles have 60 - 70 S RNA, a buoyant density of 1,186 g/ml and were shown to cross react with serum against the group specific antigen of MPMV. Morphologically they resemble the prototypes of the type B and D oncoviruses but are distinct from both. These virions are referred to as JSRV and have been shown to be biochemically, immunologically and morphologically distinct from SA - OMV I and the new viral isolate, BRV.

A table has been drawn up to summarise the biochemical, immunological and morphological features of JSRV, SA - OMV I, BRV and the prototypes of type B, C and D oncoviruses so as to facilitate a comparison between the viruses.

TABLE 16 A summary of the biochemical, immunological and morphological characteristics of JSRV, SA - OMV I and BRV so that a comparison could be made between the three viruses and the prototypes of the type B, C and D retroviruses.

BIOCHEMICAL CHARACTERISTICS

JSRV	SA-OMV I	BRV	Type B MMTV	Type C MuLV	Type D MPMV
<u>Proteins x 10⁻³</u>					
26	14	16	10	10	10
29	16	30	14	12	12
32	28	32	23	15(E)	14
38	55	45	36	15	16
50	80	74	38	30	20(E)
75-77	97	84	52	70	27
82-84	135	122			70
<u>RDP cation preference</u>					
2+	2+	2+	2+	2+	2+
Mg	Mg	Mn	Mg	Mn	Mg
<u>Density in sucrose g/ml</u>					
1,186	1,155	1,16	1,17	1,16	1,17

IMMUNOLOGICAL CHARACTERISTICS

JSRV	MV	BRV	(B) MMTV	(C) MuLV	(D) MPMV
<u>JS specific MAbs</u>					
2E3	+	-	NT	NT	-
4A10	+	-	NT	NT	-
11G11	+(P50)	-	NT	NT	-
<u>Polyclonal antibodies</u>					
MPMV	+(p29)	-	+(p32)	+(p27)	-
MV	-	+(p28)	-	NT	+(p27)

(+), represents + VE.

(-), represents - VE.

(NT), represents not tested.

MORPHOLOGICAL CHARACTERISTICS

JSRV	MVV	BRV	(B) MMTV	(C) MuLV	(D) MPMV
<u>Presence of A type particles</u>					
+	rare	+	+	+	+
<u>Nucleoid at budding</u>					
complete	incomplete	com/incom	complete	incomplete	com/incom
<u>Immature virus</u>					
rare	+	+	+	+	+
<u>Mature virus</u>					
<u>Nucleoid</u>					
slightly eccentric	eccentric	centric	eccentric	centric	centric
<u>Perinucleoidal space</u>					
dense	lucent	dense	lucent	lucent	dense
<u>Surface projections</u>					
spikes	occasional knobs	absent	spikes	short	short knobs

All three viruses possess reverse transcriptase activity. However, JSRV and SA - OMV I differ from BRV in that the latter has a manganese dependent enzyme whereas both JSRV and MVV have a magnesium requirement. The types B and D retroviruses have magnesium dependence whereas type C viruses generally have a manganese dependence (Dahlberg et al., 1980). The lentiviruses resemble the type C viruses morphologically but because they are non transforming (and have other differences) they are classified in a

separate sub - family, that of the slow viruses. Members of the type C retroviruses include the leukemia viruses such as MuLV, FeLV, BLV and many of the non - human primate oncoviruses (Lowry, 1985). Because of the morphological similarities between JSRV and the type B and D retroviruses, the serological cross - reactions between them and their common dependence on magnesium as divalent cation, JSRV was thought to belong in one of these two classes (Sharp and Herring, 1983). However, there are other differences. The polypeptide patterns are different, especially when the low molecular weight components are compared. The known type D oncoviruses are all primate viruses (Fine and Schochetman, 1978) whereas type B oncoviruses have only been isolated from mice and guinea - pigs so far. JSRV may therefore belong to a new class of oncovirus.

There is no uncertainty about the classification of SA - OMV I as a member of the sub family Lentivirinae. It has even been confirmed at the nucleotide level (Payne et al., 1986; Querat et al., 1987). Serologically it can be distinguished clearly from JSRV as shown in Chapter 4 and in hybridization experiments lentivirus probes have failed to reveal any homology with DNA isolated from JS lesions (Perk and Yaniv, 1977).

BRV is classified as a novel retrovirus because it has reverse transcriptase activity, RNA as its genetic material and a density of 1,16 g/ml, which falls within the permitted range of retrovirus densities (1,15 - 1,20 g/ml in sucrose) (Matthews, 1982). Because of the transformed nature of the cells from which it was recovered

(as demonstrated in the nude mouse experiment) BRV is regarded as a possible member of the sub family Oncovirinae. As BRV is the most likely retrovirus to be isolated from cattle it was important to determine if BRV was related to Bovine Leukemia virus (BLV). However, it was shown (Plate 26) that these two viruses are not serologically related. Morphologically BLV is also different to BRV in that the former does not have immature A type particles and it has an electron lucent perinucleoidal space (Payne, 1985) whereas in BRV the perinucleoidal space is electron dense (Payne, 1985). Recently, Amborski et al., (1987) described the isolation of a retrovirus from tissue taken from a Bison that was suffering from MCFV. This virus was shown to have a magnesium dependent reverse transcriptase and to be serologically related to bovine syncytial virus (BSV), a common foamy virus contaminant of bovine cell cultures (Matthews, 1982).

The possibility that BRV could also be related to BSV had to be considered, especially in view of the fact that MCFV was also involved in its original isolation. Unfortunately, BSV antiserum could not be obtained for a serological comparison. However, BSV is strongly cell - associated whereas BRV is found in cell - free culture medium (Amborski et al., 1987). Furthermore, BSV is non transforming whereas BRV was isolated from transformed cells. Thirdly, BSV has spikes on its membrane (Clarke et al., 1970) whereas BRV has a smooth surface membrane. It does not seem likely that there is a relationship between the two viruses, although the possibility that BRV may be a mutant of BSV containing an oncogene cannot be excluded at present.

As mentioned above the evidence is convincing that JSRV does cause JS. However, the inability to produce the virus in vitro suggests that there is something unusual about this virus. Because of its acutely transforming nature in vivo the JSRV probably contains an oncogene. It is well known that most retroviruses containing oncogenes are replication defective though active in transformation (Evans and Lennox, 1985). It is therefore possible that the JSRV requires a helper virus to replicate. Evidence is available that both supports and refutes this idea. In many repeat experiments, in this laboratory, unsuccessful attempts were made to demonstrate a helper role for SA - OMV and other retroviruses in JSRV replication. However, none with any success. Limited attempts to recover JSRV by co - cultivating JS tumour cells with BRV producing cells also failed to stimulate the growth of JSRV in vitro (personal observations)

In 1984, Irving, Perk, Hod, Gazit, Yaniv, Zimmer and Tal, claimed that they had propagated a retrovirus from sheep affected with JS that also caused JS. However, the biochemical characteristics of the virus , density between 1,15 - 1,17 g/ml and type C morphology, suggest that it was more likely MVV that they had isolated. In fact they showed that the virus had a limited genetic relatedness to the members of the lentivirus family. At a latter stage it was shown that this virus did not contain the JSRV p29 when reacted with MPMV antiserum in an immunoblot technique (DeVilliers, personal communication). Circumstances surrounding their virus isolation was very similar to that in our laboratory when SA - OMV I was isolated at about the same time. During attempts to

culture JSRV from JS lung wash material a virus which later proved to be a lentivirus was recovered (York, 1984 and Payne et al., 1986). This isolate, SA - OMV I, was injected into a number of sheep to determine whether it could cause JS. However, after two years none of the seventeen sheep developed JS (Payne et al., 1986). Since then the lentivirus has been recovered from many sheep affected with JS in our experimental sheep and throughout the world. Recently, in North America, 6 out of 7 JS sheep were shown to be infected with MVV (Demartini et al., 1987).

For several years our laboratory has investigated the possible role of the lentivirus in JS. Experiments have included concurrent infection with both viruses to attempt a reduction in the time taken for JS symptoms to develop. However, no noticeable difference was observed (Verwoerd, unpublished results). It was also attempted without success to reduce the effect of SA - OMV I by injecting the JSRV together with anti - MVV antibodies. With the advent of AIDS, which is caused by HIV -I (Human immunodeficiency virus), a human lentivirus, and is characterized by immunosuppression, the possible immunosuppressive role of SA - OMV I in JS was investigated. Immunosuppression was assayed using an avian tuberculin skin sensitivity assay. A group of 48 normal sheep, 21 SA - OMV I infected sheep and 30 JS + SA - OMV I infected sheep were used in the experiment. The results revealed that the lentivirus infected sheep were 36 % immune suppressed compared to the normals, and the JSRV + SA - OMV I infected group were 46 % immune suppressed. In other words both JS and SA - OMV

I were shown to have an immunosuppressive effect and each virus could possibly predispose the sheep to infection by the other (Verwoerd et al., 1987).

Jaagsiekte is characterized by a proliferation of alveolar macrophages (Broekman, Eksteen and Verwoerd, 1977) and MVV has been shown to multiply in these cells (Gendleman et al., 1985). A colleague has demonstrated that the increased population of alveolar macrophages in JS lungs is initiated by a chemotactic factor derived from the tumour cells (Myer, Verwoerd and Garnett, 1987). It is therefore possible that sheep with JS have an increased susceptibility to MVV infection, as suggested by Dawson, Venables and Jenkins, 1985). These workers showed that lung wash from lambs infected with both JS + MVV had a thirtyfold higher rate of infection with MVV than alveolar cells washed from experimentally infected lambs infected with MVV 4 weeks previously.

The role of the alveolar macrophages in JS has also been given a high priority in JS research in this department. It was actually shown by Broekman et al., (1977) that lambs given macrophage antiserum, to suppress the macrophage population, had a much lower incidence of JS than the controls. It is known that macrophages regulate remodeling and repair of connective tissue in lung injury through the production of soluble mediators. Alveolar macrophages by similar mechanisms may promote proliferation of alveolar type II cells (the transformed cell in JS), thereby increasing the cellular targets for JSRV (Demartini, et al., 1987). Support for such a mechanism has been obtained in a study in rats where it was

demonstrated that macrophages secrete substances that stimulate replication of the type II cells which could act as target cells for neoplastic transformation by an oncogenic retrovirus. Could the BRV cells and virus injected into the lungs of lambs stimulate a macrophage response that results in the proliferation of the type II cells which in turn activate or stimulate the JSRV. It still has to be determined whether the JSRV is an endogenous virus or whether it resides in certain sheep as a latent defective provirus.

It was very interesting to observe in the sera of the four sheep (injected with BRV) that the one sheep that developed the most chronic JS symptoms had no antibodies to BRV p32. On the other hand the sheep that did not develop JS had a higher antibody titre to that protein. The possibility of a vaccine against JSRV is always a main priority at Onderstepoort. To investigate the possible use of BRV p32 in the development of a JS vaccine an immunization programme is underway where fifteen pregnant ewes were immunized with BRV so that they could develop a high titre of antibodies to BRV p32. The idea being that when the ewes lamb they would transfer the antibodies to their offspring via their milk so that on challenge with JSRV, after birth, (the most susceptible time for JSRV infection) it is hoped that the lambs will be protected from developing JS. This experiment is at the stage where the ewes are about to lamb.

Whatever the role of BRV in JS the finding that three out of four

lambs injected with BRV developed JS is highly suggestive that BRV could play some role in JS. Even though this suggestion is based on a preliminary result it still remains that there is a serological link between the two viruses. Future work is aimed at investigating whether the BRV, which is a very practical virus to work with in that the cells and virus grow very well, could be used as a JS research tool.

The JS system is a difficult but ideal animal model for the study of human lung carcinoma. In addition the involvement of MVV, which is in the same sub family as HIV, the etiological agent of AIDS, makes all research in this system very relevant and useful as an animal model.

APPENDIX I

BUFFER B (Verwoerd, Williamson and DeVilliers, 1980)

Tris Magnesium EDTA (TME) stock solution (10 X conc)

Tris. HCl, pH 7,5	60,57 g
MgCl ₂	10,15 g
EDTA	1,86 g

Make up to one litre with sterile distilled water.

Buffer B

100 ml TME stock solution	
KCl	3,725 g
DTT	0,075 g

Make up to one litre with distilled water.

AQUEOUS SCINTILLATION COCKTAIL (Bray, 1960)

Naphthalene	60 g
PPO	4 g
POPOP	0,2 g
Methanol (absolute)	100 ml
Ethylene glycol	20 ml

Make up to one litre with p - Dioxane. This cocktail is stable for one month.

PHOSPHATE BUFFERED SALINE (PBS) (Dulbecco and Vogt, 1954).

Solution A

KCl	0,2 g
NaCl	8,0 g
Na ₂ HPO ₄ .2H ₂ O	1,44 g
KH ₂ PO ₄	0,2 G
H ₂ O	750 ml
2	

Solution B

CaCl ₂ .2H ₂ O	0,13 g
H ₂ O	250 ml
2	

Solution C

MgCl ₂ .6H ₂ O	0,1 g
H ₂ O	100 ml
2	

Add solution B slowly to solution A and then add solution C slowly to the mixture. Make up to one litre with sterile water.

TE (TRIS- EDTA) BUFFER

	100 X conc
0,1 M Tris	100 g
0,001 M EDTA	37,2 g

Adjust the pH to 7,4 and add distilled H₂O to make one litre.

Dilute 1:100 prior to use.

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ISOLATION AND IDENTIFICATION OF A SOUTH AFRICAN LENTIVIRUS FROM JAAGSIEKTE LUNGS

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ABSTRACT

PAYNE, A., YORK, D. F., DE VILLIERS, E.-M., VERWOERD, D. W., QUÉRAT, G., BARBAN, V., SAUZE, N. & VIGNE, R., 1986. Isolation and identification of a South African lentivirus from jaagsiekte lungs. *Onderstepoort Journal of Veterinary Research*, 53, 55-62 (1986)

In the course of attempts to grow the jaagsiekte retrovirus in cell culture, a typical lentivirus was isolated for the first time in South Africa from adenomatous lungs. Morphologically the virus could not be distinguished from other lentiviruses, but serologically it was shown to be more closely related to visna virus than to caprine arthritis-encephalitis virus. However, a preliminary restriction enzyme analysis of the linear proviral DNA of this new lentivirus (SA-DMVV) revealed that it is significantly distinct from visna virus and CAEV and therefore may represent a third type of lentivirus. Antibodies to the virus were demonstrated in a number of sheep in various parts of the country, but a direct link to a disease condition was not found. Attempts to produce lung lesions by intratracheal injection of the virus have been unsuccessful to date but a transient arthritis was produced by intra-articular inoculation. Viral replication seems to be enhanced in jaagsiekte lungs.

INTRODUCTION

Jaagsiekte (JS) or ovine pulmonary adenomatosis has been closely associated with maedi, a chronic progressive interstitial pneumonia, in various parts of the world. The two diseases were introduced into Iceland at the same time and possibly by the same imported animal (Pálsson, 1976). In Peru, both diseases were demonstrated in one sheep (Snyder, DeMartini, Ameghino & Caletti, 1983), while both were shown to be present in the same flock in Kenya (Wandera, 1971), the Netherlands (Houwers & Terpstra, 1984) and in England (Markson, Spence & Dawson, 1983).

In South Africa, maedi and the associated neurological affection, visna, in their classical forms have never been diagnosed. However, in two early descriptions of the histopathological lesions of jaagsiekte, reference was made to 'chronic catarrhal pneumonia' and 'interstitial fibroid changes' (Mitchell, 1915) as well as to 'thickening of interalveolar tissue' and 'lymphoid infiltration' (Cowdry, 1925), lesions which are certainly not typical of jaagsiekte. In comparing his own observations with those of these authors, De Kock as far back as 1929 concluded that there may be two specific lung diseases in sheep, a 'papilliform cystadenoma' (jaagsiekte) and a 'chronic indurative catarrhal pneumonia', which was called 'Graaff-Reinet disease' after the district in which it was first encountered. Graaff-Reinet disease has not been diagnosed since, however.

When the aetiological agent of jaagsiekte (JSRV) was shown to be a retrovirus (Verwoerd, Williamson & De Villiers, 1980), the question was immediately raised of a possible relationship to visna virus, which is the prototype of the *Lentivirinae*, a subfamily of the *Retroviridae* (Matthews, 1979).

The morphology and morphogenesis of JSRV were quite distinct, however, and no evidence for a serological relationship to visna virus (VV) was found (Verwoerd *et al.*, 1980; Payne, Verwoerd & Garnett, 1983). Furthermore, the negative results of a limited serological survey supported the contention, based on clinical and pathological observations, that maedivisna did not occur in South Africa.

The isolation of a lentivirus from the lungs of experimental jaagsiekte cases, which is reported in this paper, was therefore quite unexpected and prompted an investigation of its relationship to the other known lentiviruses,

the prototypes of which are visna virus and caprine arthritis-encephalitis virus (CAEV), as well as its possible role in disease.

MATERIALS AND METHODS

Cell cultures

Sheep choroid plexus (SCP) cells were prepared from foetal tissues, frozen away as primary cultures and subsequently used for virus isolation within 3-6 passages. Ovine foetal trachea (OFTR), ovine foetal turbinate (OFTU) and Himalayan tahr ovary (HTO) cell lines were obtained from Dr Lehmkuhl, Animal Disease Center, Ames, Iowa. All cells were maintained in Dulbecco's minimal essential medium supplemented with high glucose, glutamine and 10 % foetal calf serum.

Lung rinse pellets

Lung rinse pellets were prepared from both jaagsiekte and normal lungs as previously described (Verwoerd, Payne, York & Myer, 1983). Briefly, 1-2 litres of cold tissue culture medium without serum was poured into the trachea and decanted after a gentle massaging of the lungs. Cells were removed from the rinse fluid by low speed centrifugation and used for establishing macrophage and primary lung cell cultures. The supernatant was pelleted in a Spinco Ti 15 batch rotor at 30 000 rpm for 90 minutes. The pellet was collected, stored at -70 °C if necessary, and further purified by passing through a 30 × 1.6 cm column of Sephacryl S-1000 superfine (Pharmacia).

Infection of cells

Virus was isolated by either co-cultivating the macrophages obtained from the lungs with SCP cells, or by infecting the cells with the semi-purified lung rinse pellets. In the latter case the cells were pretreated for 1 hour with polybrene at 8 µg/ml before adding the purified pellet in medium containing the same concentration of polybrene. Adsorption was allowed to take place for 3-4 hours.

Infected cell cultures were monitored for virus replication by looking for cytopathic effects (cell fusion and rounding of cells) and by assaying the supernatant at weekly intervals for reverse transcriptase (RDP) activity. The RDP assay has been described previously (Verwoerd *et al.*, 1983).

Polyacrylamide gel electrophoresis

RDP-positive supernatants were pelleted at 30 000 rpm for 90 min in a Spinco rotor 30 and the pellets dissociated by heating for 3 min in a boiling water bath in 2.5 % SDS, 5.0 % β-mercaptoethanol and 12.5 %

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glycerol. Proteins were then fractionated electrophoretically in a 0,1 % SDS-containing 12,5 % polyacrylamide gel with a 4 % stacking gel according to King & Laemmli (1971).

Immunoblot assay

Protein-SDS bands were transferred from the polyacrylamide gels to nitrocellulose sheets (S&S) in a Bio-Rad electroblot apparatus in a 20 % methanol containing tris-glycine transfer buffer at pH 8,3. Transfer was usually carried out at 0,45 amps for 4 hours at 4 °C. For immunological detection of the transferred proteins, the nitrocellulose was cut into the desired strips and blocked in 50 % horse serum in PBS for 1 hour with gentle rocking. The strips were then incubated overnight with the relevant primary antiserum diluted 1:20 in 50 % horse serum. Excess antiserum was removed by rinsing 3 times in washing buffer (0,025 % Tween 20 in PBS) and the strips then incubated for 1 hour in peroxidase-conjugated antibody against sheep IgG. After repeating the washing procedure, freshly prepared peroxidase substrate (0,06 g 4-chloro-1-naphthol in 20 ml methanol + 100 ml PBS + 60 μ l H₂O₂) was added. When the bands were clearly visible the reaction was stopped by immersing the strips into deionized water for 10 min and drying between filter paper.

In all the experiments low molecular mass markers (Pharmacia), stained with amido black, were run concurrently with the samples, which included positive serum/antigen controls as required.

Antisera

Sheep anti-visna virus serum was obtained from Dr D. J. Houwers, Lelystad, Netherlands, and used at a 1:50 dilution. Goat anti-Mason-Pfizer monkey virus p27 was supplied by the National Cancer Institute, Bethesda, Maryland and used at a 1:60 dilution. Goat anti-CAEV serum was obtained from Dr O. Narayan, Baltimore, USA and used at 1:100. Goat anti-sheep IgG was obtained from Dakopatts, Copenhagen, Denmark, and used at a dilution of 1:400.

Restriction enzyme digestion, Southern analysis and molecular hybridization

Unintegrated proviral DNAs were selectively extracted from lentivirus-infected cells by the Hirt procedure and digested with restriction enzymes. The digested and undigested DNAs were analyzed electrophoretically in 1 % agarose gels, transferred to nitrocellulose, and hybridized to a ³²P nick-translated DNA of lambda vis 109 visna virus insert as previously described (Quérat, Barban, Sauze, Filippi, Vigne, Russo & Vitu, 1984).

Electron microscopy

Samples were prepared for electron microscopy as described previously (Payne *et al.*, 1983) and viewed in a Jeol transmission electron microscope.

Immunoperoxidase staining technique

An anti-visna virus peroxidase conjugate was made according to Avrameas (1969). Frozen sections of jaagsiekte lungs were fixed in 96 % ethanol, blocked for 10 min with 3 % bovine serum albumin in PBS and then incubated with the conjugate diluted 1/50 in 3 % bovine serum albumin in PBS. After incubating for an hour at room temperature the sections were washed with 0,5 % Tween 20 in PBS. The substrate (10 mg 3,3'-diaminobenzidine tetrahydrochloride¹ in 20 ml of PBS containing 0,01 % H₂O₂) was added to the sections for 10 min. The sections were washed under running tap water stained for 1 min with Haematoxylin and mounted.

RESULTS

Initial isolation

The original discovery of a South African lentivirus was made during attempts to cultivate jaagsiekte retrovirus (JSRV) in sheep choroid plexus (SCP) cells. A lung rinse pellet, which was obtained from an experimental case of jaagsiekte as previously described (Verwoerd *et al.*, 1983), was purified by gel filtration as described under Materials and Methods, without freon pretreatment. Virus concentrations in the eluted fractions were determined by means of a reverse transcriptase (RDP) assay. A combination of RDP-positive peaks was used to infect polybrene-treated secondary SCP cells (see Materials and Methods). RDP-activity was first detected in cell culture supernatants on Day 17 and reached a peak

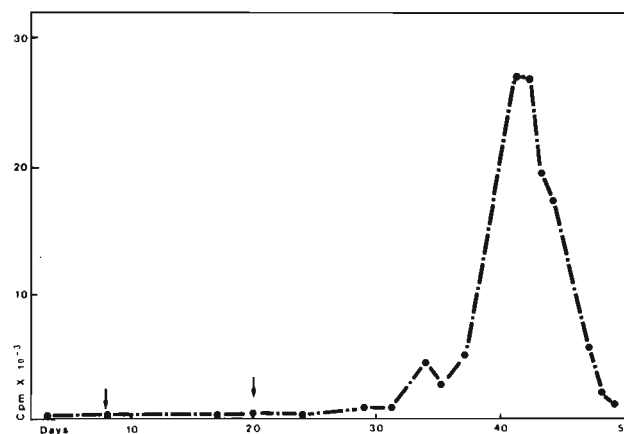


FIG. 1 Replicative cycle of SA-OMVV in SCP cells. Virus production is measured in terms of RDP activity (cpm)

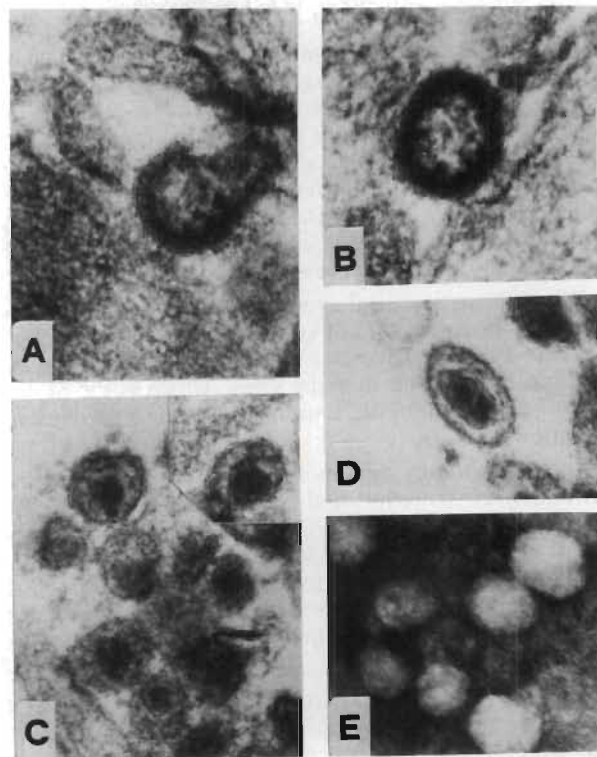


FIG. 2 Electron micrograph of lentivirus produced in sheep choroid plexus cells. Bar = 100 nm

- 2A Budding lentivirus particle
- 2B Immature virion
- 2C Mature virion
- 2D Multinucleoid lentivirus particle
- 2E Negatively stained lentivirus

¹ Sigma Chemical Company, St. Louis, MO, USA

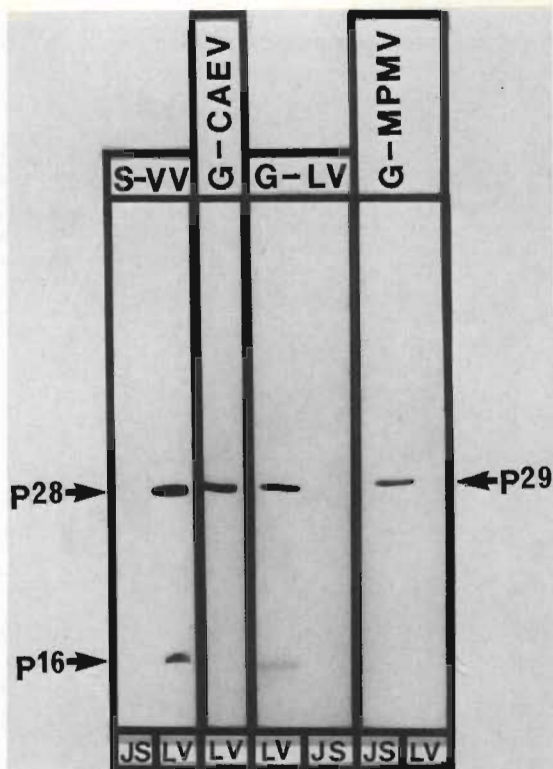


FIG 3 An immunoblot result showing the p28 and p16 antigens of the novel lentivirus (LV) which are detected by both sheep antiserum to visna virus (S-VV) and goat antiserum to the lentivirus (G-LV). These 2 sera do not detect any proteins in the JSRV (JS) lanes. The goat antiserum to CAEV (G-CAEV) detects a p28 in the novel lentivirus (LV) only. A goat antiserum to MPMV (G-MPMV), which is JSRV specific, detects a p29 in the JSRV (JS) lane, but does not react with any antigens of the lentivirus (LV).

on Day 41 after infection (Fig. 1). At this stage typical cytopathic effects were seen, consisting of syncytium formation and dying cells. The retrovirus, which is produced by these cells and was first thought to be JSRV, therefore has a very long replicative cycle, albeit quite variable in different cell types (see below).

Identification as a lentivirus

The first indication that the virus replicating in SCP cells was not JSRV was obtained by electron microscopic examination. The morphogenesis of the virus was quite distinct from that of JSRV (Payne *et al.*, 1983) and resembled that described for visna virus. The virus budded with a crescent shaped core with no intermediate space (Fig. 2A) to form immature particles with an electron-dense layer immediately below the viral envelope (Fig. 2B). Mature particles were membrane-bound with eccentric cores and in some particles a clear intermediate layer was visible (Fig. 2C). Multinucleoid particles were also observed (Fig. 2D). On some negatively stained particles there appeared to be knobs on the surface, but on most particles there was no distinct surface structure (Fig. 2E). These particles were quite distinct from those described for JSRV.

The buoyant density of the virus, determined by isopycnic centrifugation in a sucrose gradient, was 1.15. This provided additional evidence that the virus rescued in SCP cells was different from the JSRV present in the lung fluid, which had a density of 1.175.

A serological relationship between the newly isolated virus and visna virus was first detected by means of an ELISA test (results not shown). As the various members of the lentivirus family could not be distinguished by this

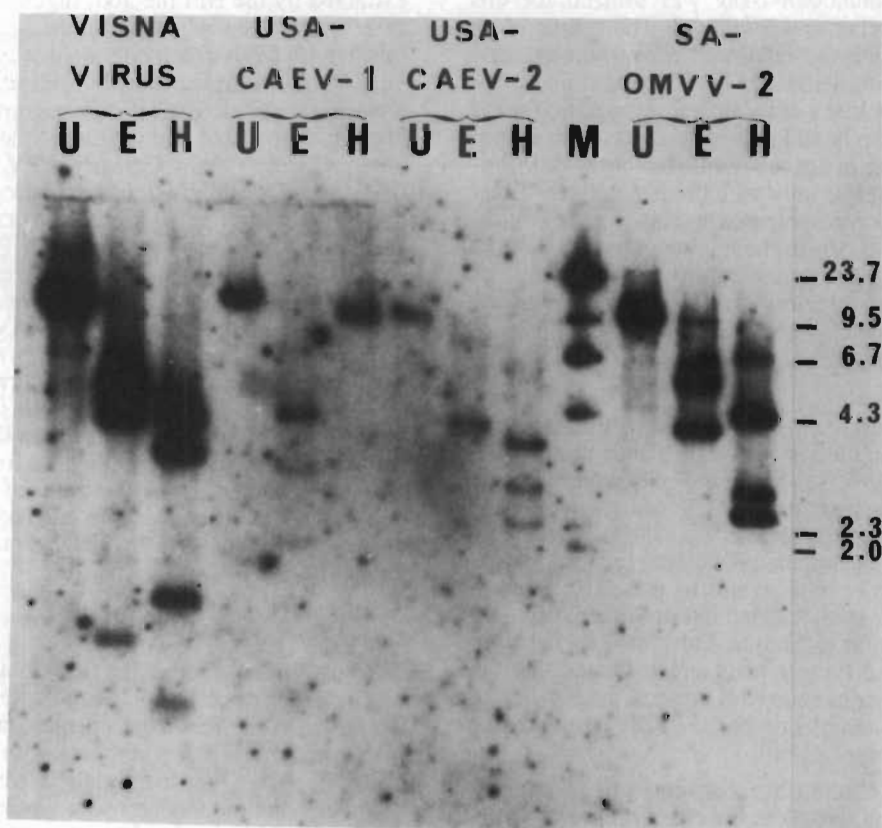


FIG. 4 Proviral DNA analysis of four lentiviruses, visna virus, USA-CAEV-1 (strain Cork), USA-CAEV-2 (strain Crawford), and SA-OMVV-2. SCP cells were infected by the Icelandic strain K1514 of visna virus and the novel SA-OMVV-2, and caprine fetal synovial membrane cells were infected by USA-CAEV-1 (CO) and USA-CAEV-2 (CR). Unintegrated proviral DNAs were extracted from the infected cells by the Hirt procedure and digested by EcoRI (lanes E), Hind III (lanes H) or were not digested (Lanes U). The digested and undigested DNAs were analyzed in 1 % agarose gel, transferred to nitrocellulose, and hybridized to a 32 P nick-translated DNA of lambda vis 109 visna virus insert. (M) Hind III-digested fragments of lambda phage DNA. Sizes (kbp) of the DNA fragments are indicated on the side of the panel.

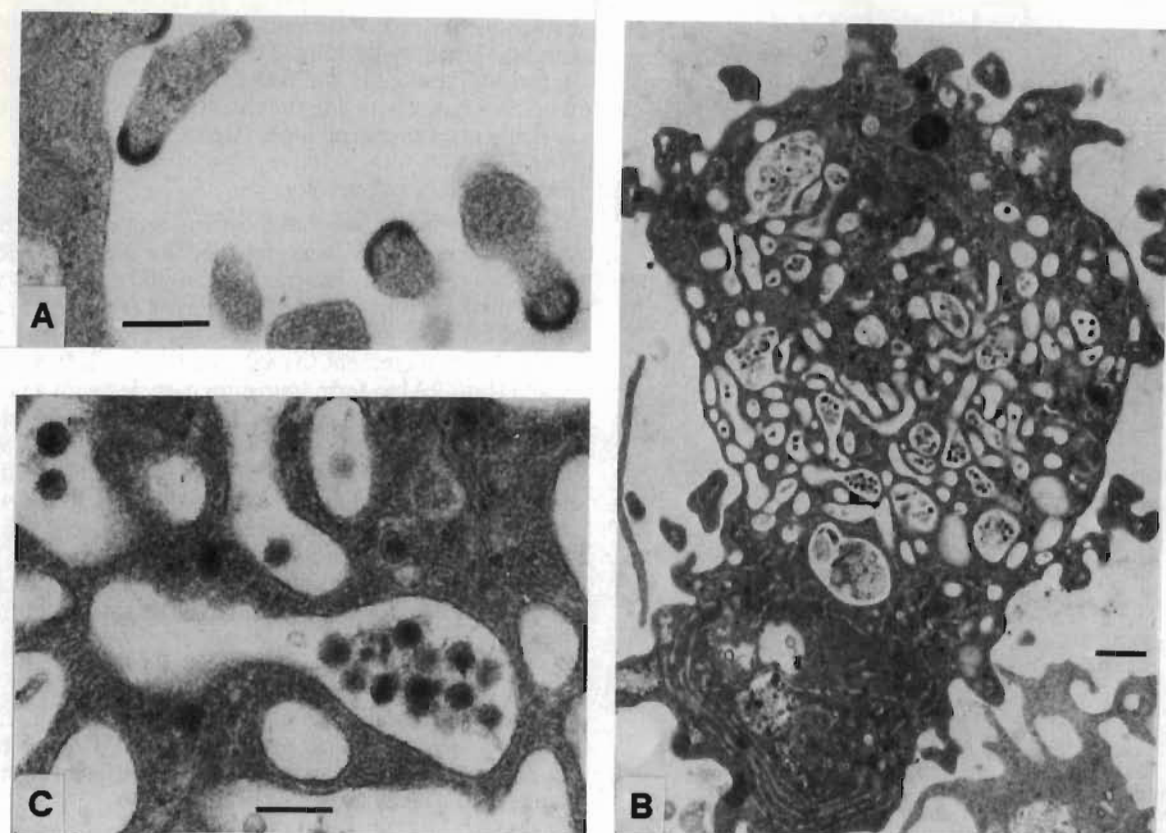


FIG. 5A Budding lentivirus particles in a 5-day-old primary cell culture from JS lungs. Bar = 200 nm
 5B Macrophage-like cell with many vacuoles containing mature lentivirus particles in the same cell culture. Bar = 700 nm
 5C Higher magnification of vacuoles described in 4B. Bar = 700 nm

technique, an immunoblot-assay was utilized for this purpose (see Materials and Methods). The results of an experiment in which the relationship between the new isolate, JSRV, visna virus and CAEV was studied, are shown in Fig. 3. A visna virus antiserum specific for the 2 internal proteins p28 and p16 cross reacts with 2 antigens of identical size in the new virus, but not with JSRV. CAEV antiserum reacts only with the p28 antigen. Likewise, a goat antiserum prepared against the new virus reacts with p28 and p16 in the homologous reaction but not with JSRV. An antiserum against the p30 protein of Mason-Pfizer monkey virus (MPMV), which was recently shown to react with a JSRV antigen (Sharp & Herring, 1983) does not react with either of the lentiviruses.

It is clear, therefore, that the new isolate, subsequently called South African ovine maedi-visna virus, isolate 1 (SA-OMVV-1) shares the p28 group-specific antigen with visna virus and CAEV and the p16 visna virus specific antigen (Quérat *et al.*, 1984), suggesting that SA-OMVV-1 is antigenically closer related to visna virus than to CAEV, with regard to these two internal proteins. A similar study carried out in France, using an immuno-precipitation technique and radio-labeled viral proteins, confirmed these results and indicated that the new lentivirus contains four viral proteins similar in size to those of visna virus, i.e. gp135, p28, p16 and p14 (Barban *et al.*, in preparation).

The antigenic relationship between gp135 of SA-OMVV-1 and visna virus was not clearly resolved using either technique, however. A restriction enzyme analysis of the proviral DNAs present in lentivirus-infected cells revealed that the nucleic acid sequences of the new lentivirus are distinct from those of visna virus as well as from those of CAEV. Briefly, unintegrated proviral DNA from cells infected by various lentiviruses was

extracted by the Hirt method, digested by restriction enzymes and electrophoresed in an agarose gel. After the transfer of DNA fragments onto a nitrocellulose filter, viral DNA was detected by hybridization with ^{32}P cDNA representative of visna virus genome (strain K1514). Fig. 4 shows that unintegrated linear DNA of visna virus, CAEV (strain Cork), CAEV (strain Crawford, G63) and SA-OMVV-2 (isolated from the tumour cell line 15.4) are all ± 10 kilobase pairs (kbp) long (lanes U). However, after digestion with EcoRI (lanes E) or Hind III (lanes H), the fragments generated from the full-length linear DNAs were distinct for the four examined lentiviruses, suggesting that SA-OMVV is a novel virus distinct from visna virus as well as two strains of CAEV. Liquid hybridization experiments between viral genomic RNA extracted from the four viruses and ^{32}P cDNA specific for each of them confirmed that the South African lentiviruses are clearly distinct from visna virus and CAEV. In contrast, the two strains of CAEV, which had dissimilar restriction enzyme maps, were found to be closely related by liquid hybridization (Barban *et al.*, in preparation).

Subsequent isolations

To determine whether the first isolation of SA-OMVV was a chance event or not, further attempts were made to isolate the virus from experimental jaagsiekte cases, normal animals and a few animals with pneumonic symptoms. Initially, lung rinse pellets were inoculated onto SCP cells and the medium was assayed for RDP activity as described above, but it was soon found that co-cultivation of lung macrophages or primary lung cultures with SCP-cells gave more consistent results. As Table 1 shows, positive isolations were made from 18/20 experimental jaagsiekte animals, including a few that did not develop tumours but had lesions of mild interstitial pneu-

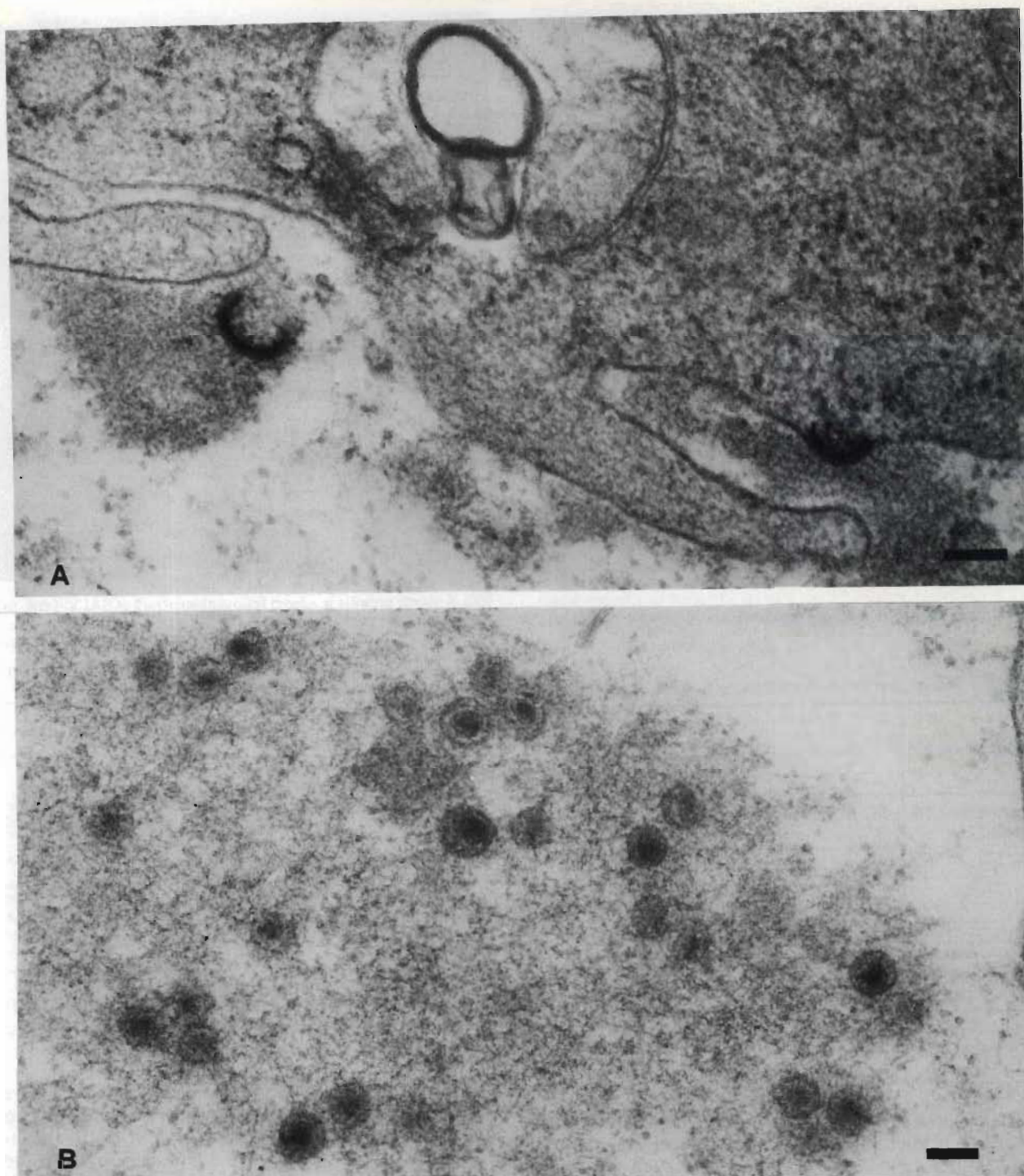


FIG. 6A Lentiviruses budding from a macrophage in a jaagsiekte lung. Bar = 100 nm
6B Mature lentivirus-like particles in the alveolar lumen of a jaagsiekte lung. Bar = 100 nm

monia. Five isolations were made from normal animals, one of which had been in contact with jaagsiekte cases for some months. Primary isolations were mostly made in SCP cells, but viral replication was also demonstrated in an SCP cell line (SCP-IIB), an ovine foetal trachea (OFTR), ovine foetal turbinate (OFTU) and Himalayan tahr ovary (HTO) cell lines. The identity of the isolates was confirmed by detecting the p28 and p16 antigens by means of the immunoblot assay using SA-OMVV-1 antiserum (results not shown) and by means of electron microscopy (Fig. 5). No differences could be detected between the different isolates.

Lentivirus particles were also observed budding from cells in primary cultures (Fig. 5A) and co-cultures. In some cases, large numbers of mature lentivirus particles

were observed in the vacuoles of these cells (Fig. 5B & Fig. 5C) and also associated with cell debris.

In vivo localization of lentivirus replication

It was possible to identify budding lentivirus particles electron microscopically in 3 jaagsiekte tumours. These particles appeared to be budding from macrophages into the alveolar lumen in close proximity to tumour cells (Fig. 6A). The infected macrophages had abnormal endoplasmic reticulum and rounded pseudopodia indicating that the virus was affecting the metabolism of these cells. Mature lentivirus particles were observed in macrophage vacuoles as well as in the alveolar lumina (Fig. 6B).

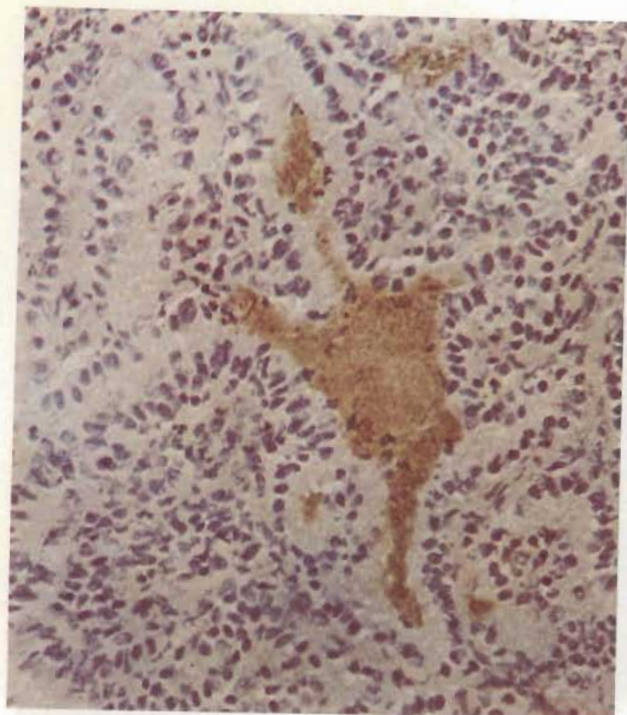


FIG 7 Lentivirus antigen (brown staining) detected in a frozen jaagsiekte lung section using a direct immunoperoxidase technique

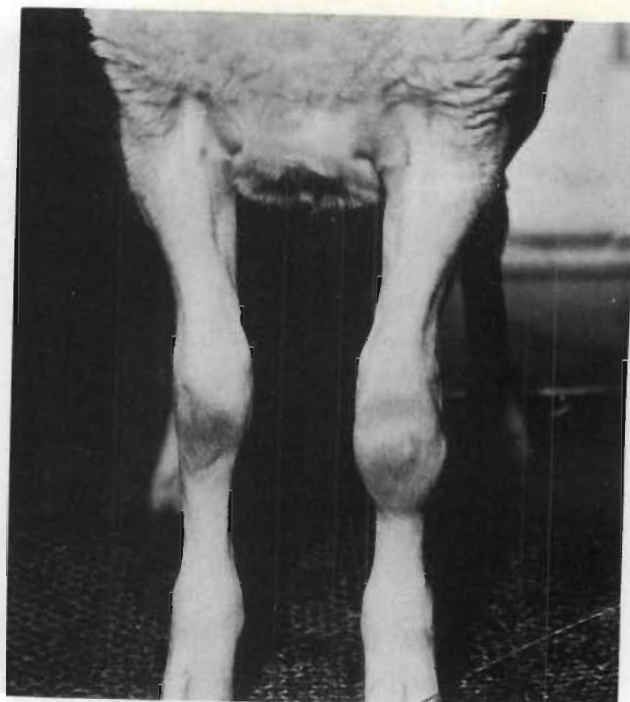


FIG 8 Acute arthritis in a lamb following injection of SALV-1 into the left knee joint

TABLE 1 Independent isolations of lentivirus from sheep lungs

Source of animals	Lung lesions	Number of positives Number of attempts	Immunoblot positives
Experimental JS cases	JS + pneumonia	7/9	4/5
	JS only	7/7	4/5
	Pneumonia only	4/4	2/3
Total		18/20	10/13
Field case of pneumonia	Chronic purulent pneumonia	0/1	—
Normal sheep:			
JS contacts	None	1/2	NT
No contact	None	4/13	4/4

The electron microscopy results were confirmed by a direct immunoperoxidase staining technique using anti-visna virus serum to detect lentivirus antigen in jaagsiekte lungs. Lentivirus antigen was localized in alveolar lumina in close association with macrophages and was often adjacent to tumour lesions (Fig. 7).

Distribution

The results of our isolation attempts suggested that SA-OMVV-1 has been co-transmitted with JSRV during the course of our studies on the aetiology of jaagsiekte. It was indeed also possible to rescue lentivirus from our 15.4 tumour cell line, (SA-OMVV-2, unpublished results), which was established in 1975 from a jaagsiekte case and formed the basis of our jaagsiekte transmission studies. It was not surprising, therefore, that 23/27 sera from our experimental jaagsiekte animals older than 2 months gave positive results when tested for antibodies to SA-OMVV, using the immunoblot assay (Table 2). To obtain some information on the distribution of the lentivirus outside our own flock, sera were collected from clinically normal sheep from various parts of the country and screened for antibodies to SA-OMVV in the

TABLE 2 Incidence of antibodies to SA-OMVV-1 p28 in randomly collected sheep and goat sera

Source of sera	Number positive/ number tested	% positive
Experimental jaagsiekte cases <2 months old.....	0/6	0
>2 months old.....	23/27	85.2
Normal sheep:		
Allerton.....	0/51	0
Potchefstroom.....	3/30	10.0
Dundee.....	0/12	0
Bloemfontein.....	1/74	1.4
Smithfield.....	26/89	29.2
Milk goats:		
Stellenbosch.....	0/18	0

same way. The results, which are summarized in Table 2, indicate that the virus is widely distributed, but its incidence on different farms can vary from 0–30 %.

In a previous study a large number of goat sera were tested for antibodies to CAEV using immunodiffusion and ELISA techniques (Adams *et al.*, 1984). The negative results of that study were confirmed for 10 of the sera which were also included in the present series. Although goats can be experimentally infected with SA-OMVV (see below), no antibodies have so far been detected in normal goat sera.

Pathogenicity

As was mentioned above, mild interstitial pneumonia was found histologically in experimental animals injected intratracheally with lung rinse material obtained from adenomatous lungs. Similar lesions were found in animals that developed tumours and in those that did not.

In an attempt to prove that these lesions were caused by the contaminating lentivirus, SA-OMVV, isolated and cultivated in cell culture as described above, was injected intratracheally into a group of 18 new-born lambs and 2 goat kids. No lesions were found in 5 lambs necropsied after 3 months or in 5 after 12 months, and no symptoms were observed after 2 years in the surviving animals, except for one sheep which developed a mild

arthritis in both carpal joints. No virus could be isolated from the synovial fluid, however. All the animals showed a positive seroconversion.

To explore further the possibility that SA-OMVV may play a role in ovine arthritis analogous to that of CAEV in goats, 2 lambs and 2 kids were injected with 10^6 RDP units of SA-OMVV into the left carpal joint. One animal in each group developed an acute arthritis with considerable swelling after 6 weeks (Fig. 8). The condition persisted for about 3 weeks and then disappeared before virus isolation was attempted.

DISCUSSION

The isolation of a lentivirus from adenomatous lungs is not too surprising in view of the known association between jaagsiekte and maedi in various countries (Malmquist, Krauss, Moulton & Wandera, 1972; Cutlip & Young, 1982; Irving, Perk, Hod, Gazit, Yaniv, Zimmer & Tal, 1984). It was unexpected, though, because maedi or the related progressive pneumonia syndrome as found elsewhere had not been diagnosed in South Africa, with the possible exception of the so-called 'Graaff-Reinet disease', described more than half a century ago.

The nature of the association between the 2 diseases is not known. Unlike experimental visna virus infections, where virus replication is restricted *in vivo* (Geballe, Ventura, Stowring & Haase, 1985), in animals infected with both jaagsiekte and lentivirus there appears to be active replication of the lentivirus in the lungs. It was recently demonstrated that visna virus can be transmitted efficiently to a natural case of jaagsiekte (Dawson, Venables & Jenkins, 1985) suggesting that adenomatous lungs are more susceptible to the infection than normal lungs. These authors also demonstrated efficient spread of visna virus from this case to other animals, suggesting active virus production. This could be a manifestation of the known susceptibility of jaagsiekte lungs to various secondary infections. An interesting speculation in this regard is the possibility that JSRV may have an immunosuppressive effect, similar to that of the Mason-Pfizer monkey virus to which it is related. Another possibility is related to the known accumulation of macrophages in jaagsiekte lungs (Tustin, 1969). Lentiviruses were found to occur in a latent form in monocytes and to be activated during maturation of these cells to macrophages (Narayan, Kennedy-Stoskopf, Sheffer, Griffin & Clements, 1983). Our demonstration that SA-OMVV can best be isolated from 1–2-week-old alveolar macrophage cultures suggests a similar site for its replication, and may explain why adenomatous lungs seem to be particularly susceptible to the virus.

A third possibility is based on the demonstration that lentiviruses can be naturally present in a latent form in long term ovine fibroblasts and can be induced by superinfection with CAEV (Barban, Quérat, Sauze, Filippi, Vigne, Russo & Vitu, 1984). It is conceivable that JSRV could play a similar role *in vivo* in activating a latent lentivirus. Whatever the explanation for the apparent synergism between SA-OMVV and JSRV, the existence of such a phenomenon is supported by our observation of mild interstitial pneumonic lesions in co-infected lungs and our inability to produce similar lesions experimentally by lentivirus infection only.

The physical characteristics of the isolated virus, such as size, density, morphology and morphogenesis are typical for lentiviruses. Unequivocal proof of its identity as a lentivirus was provided by the serological demonstration that it shares the p28 group-specific antigen with both visna virus and CAEV. A p16 antigen is shared only with MVV, suggesting a close relationship to this

virus. However, Southern blot hybridization experiments indicated that SA-OMVV is distinct from visna virus and CAEV and thus could represent a third type within the lentivirus group (Barban *et al.*, in preparation).

The origin of the isolated lentivirus is uncertain. Isolations were consistently made from experimentally produced JS cases. These animals were all injected with material obtained from previous cases, however, suggesting an efficient co-transmission with JSRV over a number of years. Antibodies to SA-OMVV-1 were indeed found in a number of the serially produced cases, and virus could be rescued from the 15.4 tumour cell line with which the serial transmission started. A limited serological survey proved that SA-OMVV infection is not limited to our experimental flock, however. The incidence of antibodies to the virus in the flocks we investigated varied between 0 and 30 %, and positive reactors were geographically wide-spread.

No definite role in any disease of economic importance can be assigned to the virus at present. It seems to have a very low pathogenic potential, similar to some of the ovine progressive pneumonia viruses (PPV) isolated in the USA. The experimental production of arthritis may indicate a possible involvement with this condition in nature, but similar results have been obtained with CAEV (Banks, Adams, McGuire & Carlson, 1983) and PPV (Oliver, Gorham, Perryman & Spencer, 1981) in lambs. As has already been discussed, possible involvement in chronic pneumonia was only observed in conjunction with jaagsiekte.

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EXPERIMENTAL TRANSMISSION OF JAAGSIEKTE (OVINE PULMONARY ADENOMATOSIS) TO GOATS

R. C. TUSTIN⁽¹⁾, ANNA-LISE WILLIAMSON⁽²⁾, D. F. YORK⁽²⁾ and D. W. VERWOERD⁽²⁾

ABSTRACT

TUSTIN, R. C., WILLIAMSON, ANNA-LISE, YORK, D. F. & VERWOERD, D. W., 1988. Experimental transmission of jaagsiekte (ovine pulmonary adenomatosis) to goats. *Onderstepoort Journal of Veterinary Research*, 55, 27-32 (1988).

Jaagsiekte was successfully transmitted to at least 2 out of 6 goats inoculated intratracheally with partially purified jaagsiekte retrovirus. Multiple, small, well circumscribed nodules found in the lungs consisted of typical papilliform proliferations of neoplastic Type II epithelial cells. Histological evidence of a mild interstitial pneumonia in 4 of the experimental animals can probably be attributed to a contaminating lentivirus in the jaagsiekte retrovirus preparation, as suggested by the seroconversion of the animals.

INTRODUCTION

Early reports on the natural occurrence of jaagsiekte (JS) in goats (Nobel, 1958; Cuba-Caparo, De la Vega & Copaira, 1961; Rajya & Singh, 1964) were controversial because of the paucity of published data and inadequate differentiation between alveolar epithelialization and true adenocarcinomatous lesions (Stamp & Nisbet, 1963; Tustin, 1969). Although later reports (Stefanou, Tsangaris & Lekkas, 1975; Banerjee & Gupta, 1979; Sriraman, Pao & Naidu, 1982) provided more convincing evidence of a very low incidence of the natural disease in goats and Sharp, Angus, Jassim & Scott (1986) have experimentally transmitted the disease to a goat kid, it was though necessary to confirm the susceptibility of goats by experimental transmission.

The experimental transmission of JS to sheep has been widely reported (Tustin, 1969; Wandera, 1971). By concentrating and partially purifying the virus inoculum and inoculating new-born lambs with the inoculum, the efficiency of transmission was greatly increased (Verwoerd, Williamson & De Villiers, 1980; Sharp, Angus, Gray & Scott, 1983). The presence of a contaminating lentivirus in the crude jaagsiekte retrovirus (JSRV) strain used in these studies may have contributed to the enhanced efficiency (Payne, York, De Villiers, Verwoerd, Querat, Barban, Sauze & Vigne, 1986).

The above techniques and materials were used in the present study in which the successful transmission of JS to new-born goats proved the susceptibility of this species to the disease.

MATERIALS AND METHODS

Experimental animals

Six goat kids of indigenous (boerbok) origin were inoculated intratracheally with 4 ml of a JSRV suspension between 2 and 7 days after birth. They were housed as a group under semi-isolated conditions, well separated from other experimental animals, and kept under daily observation.

Inoculum

The JSRV suspension was obtained from the lungs of an experimental case of jaagsiekte which formed part of a serial transmission study in sheep. The sheep's lungs were rinsed with 1 500 ml of cold tissue culture medium (Minimal Eagle's Medium) which was then centrifuged for 90 min in a Beckman Ti-15 batch rotor at 100 000 × g. The sediment was resuspended in about 24 ml of PBS and stored before use for 14 weeks at -70 °C. Immediately before inoculation it was thawed out and extracted

once with cold Freon 113 (Dupont). Each kid received 0.36×10^6 RDP units of virus (Verwoerd, Payne, York & Myer, 1983). By comparison, the same dose of virus produced advanced jaagsiekte lesions in newborn lambs within 2½ months (Verwoerd, Tustin & Payne, 1985).

Preparation of lung samples

Necropsies were performed and lung specimens were fixed in buffered formalin and further processed for histological examination, using standard techniques. For transmission electron microscopy (TEM), samples were prepared as described previously (Payne, Verwoerd & Garnett, 1983) and viewed with a Joel transmission electron microscope.

Serology

Serum was collected from each animal before inoculation and from 4 others at the time of slaughtering. Sera were tested for antibodies to the p28 and p16 antigens of the South African isolate of lentivirus by means of an immunoblot technique described previously (Payne *et al.*, 1986).

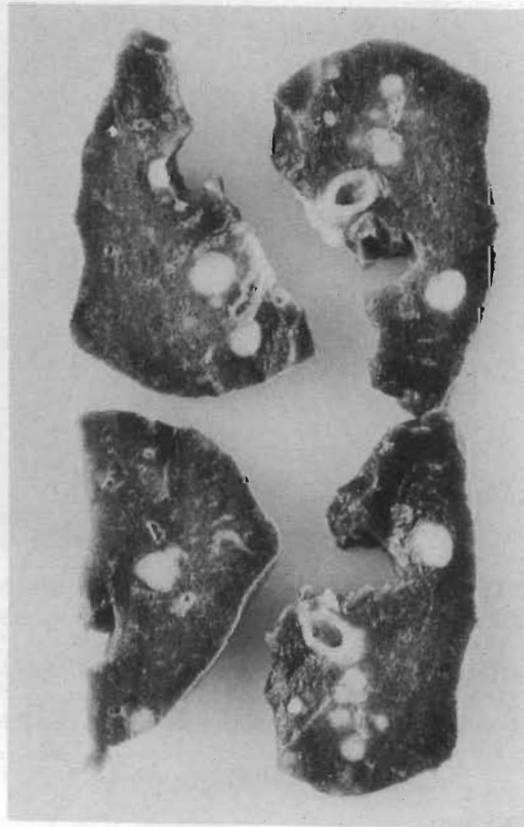


FIG. 1 Multiple, discrete, white nodules 2-7 mm in diameter, of jaagsiekte lesions in the lungs of Goat 2 after formalin fixation

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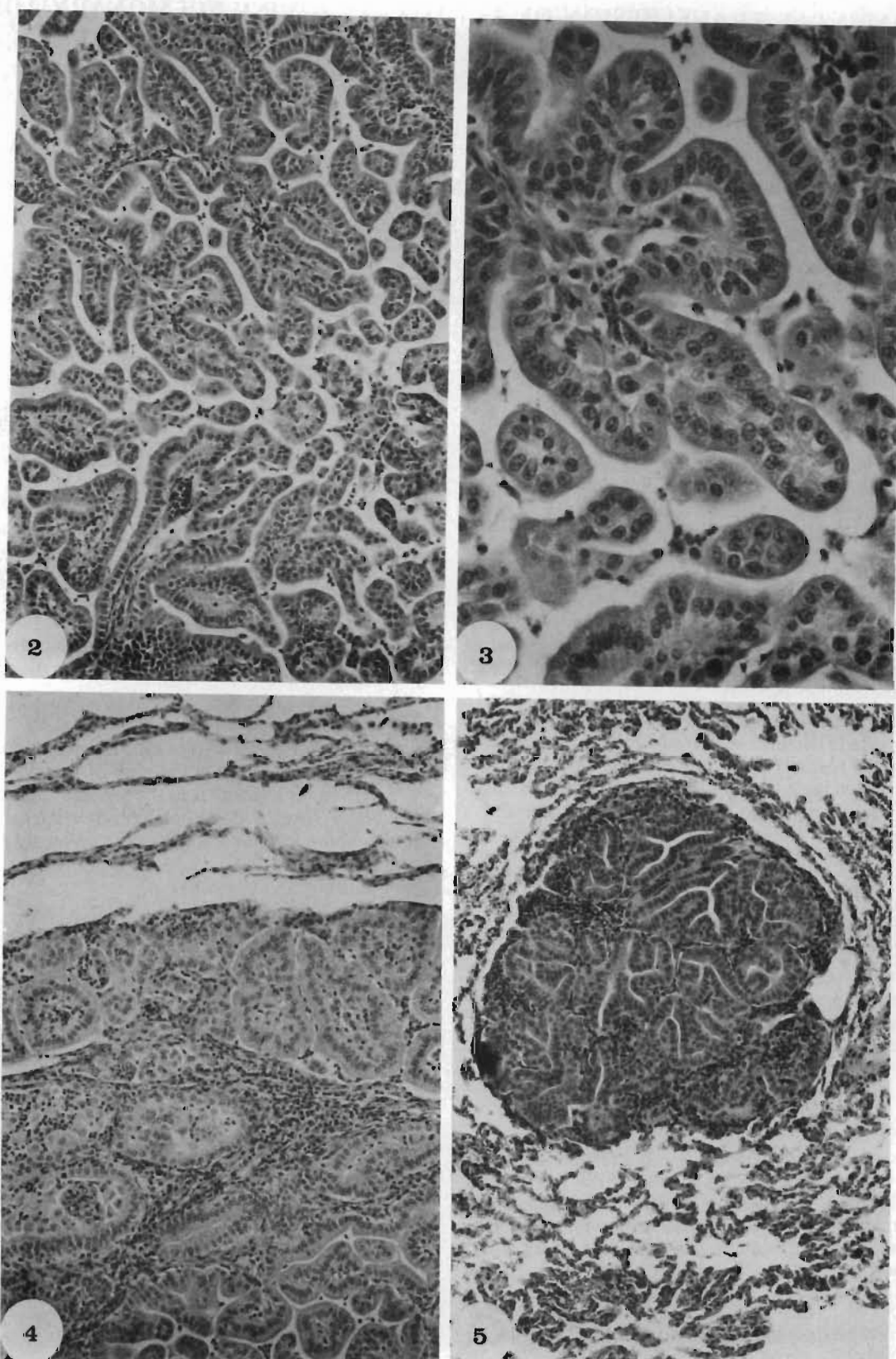


FIG. 2 Goat 3: Bronchiolo-alveolar adenocarcinoma identical with that of ovine jaagsiekte. HE \times 120

FIG. 3 Well-differentiated cuboidal to columnar neoplastic epithelial cells supported by a delicate fibrous connective tissue stroma in the lungs of Goat 3. HE \times 300

FIG. 4 The edge of one of the tumour nodules in Goat 3. Many of the lesions were infiltrated by neutrophils (smaller dark-staining nuclei) with no surrounding fibrous capsule. HE \times 120

FIG. 5 A section through a small intact tumour nodule in Goat 2. HE \times 120

RESULTS

Clinical signs

One of the 6 kids (Goat 1, see Table 1) died at the age of 5 months without developing any respiratory symp-

toms. Death was caused by acute coccidiosis. Another (Goat 2) developed nervous signs after 9 months. Necropsy revealed an acute meningitis but also multiple solid nodules in the lung. A 3rd animal (Goat 3) showed mild dyspnoea and coughing, suggesting possible jaag-

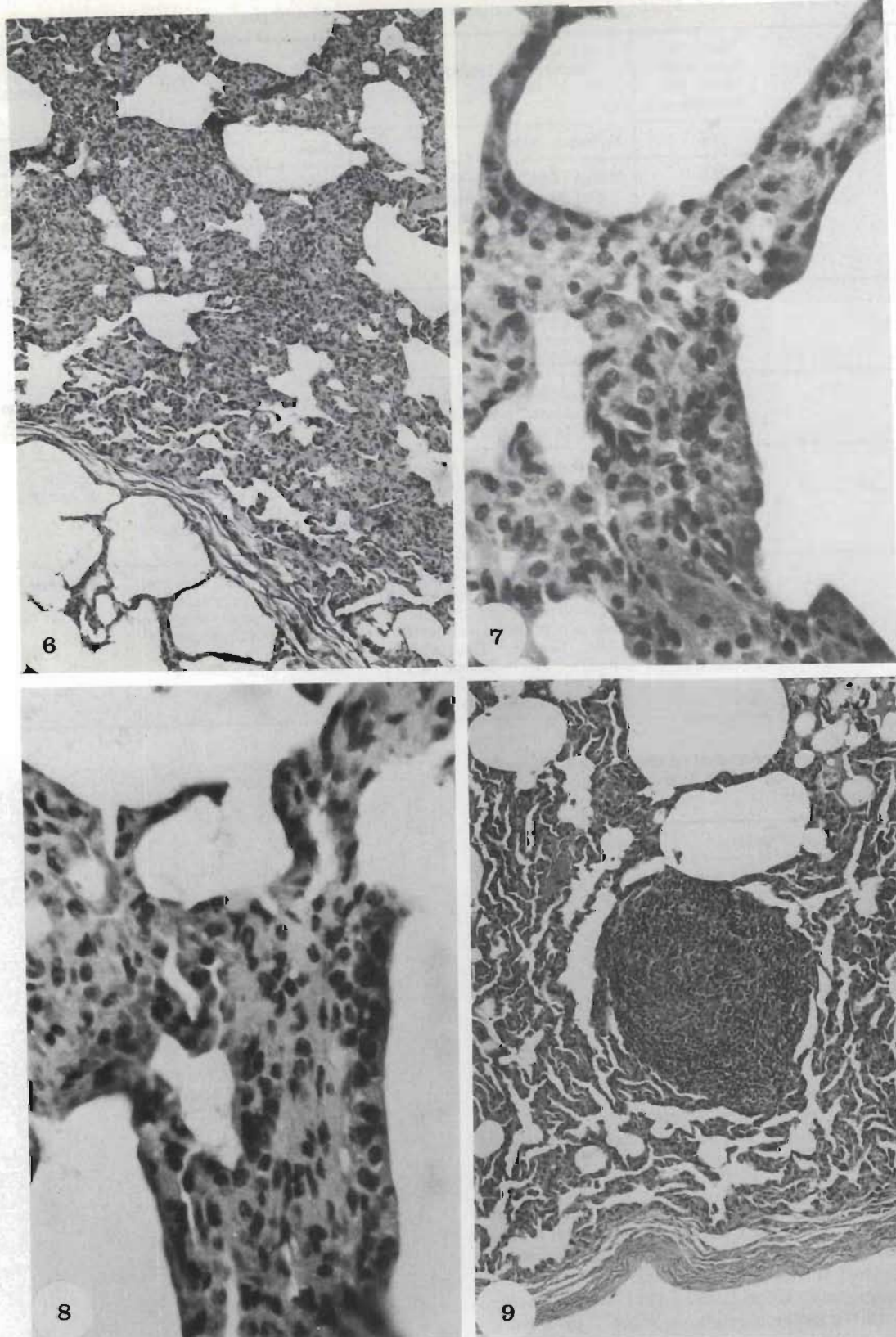


FIG. 6 A lobule in the lung of Goat 5 exhibiting interstitial pneumonia adjacent to a normal lobule. HE \times 120

FIG. 7 Interstitial pneumonia in the lungs of Goat 2 manifested by a thickening of alveolar walls caused by an infiltration principally of macrophages and lymphocytes and a reduction in the size of alveolar spaces. HE \times 480

FIG. 8 "Epithelialization" or pneumocyte Type II proliferation present in some of the areas exhibiting interstitial pneumonia, represented here also by a thickening of alveolar walls by macrophage and lymphocyte infiltration and connective tissue proliferation of Goat 5. HE \times 480

FIG. 9 A section through a nodule of lymphoid hyperplasia in the lungs of Goat 6. HE \times 120

siekte, at the age of 13 months. The other 3 did not develop any clinical signs before they were slaughtered.

Pathology

A summary of the most salient features of the pathology is presented in Table 1.

In the lungs of Goats 2, 3 and 5, macroscopically visible lesions resembling jaagsiekte were evident. These were most obvious in Goat 3 and comprised mil-
iary, discrete, firm, grey-white opaque nodules varying in diameter from 1–10 mm which were most numerous

EXPERIMENTAL TRANSMISSION OF JAAGSIEKTE TO GOATS

TABLE 1 Summary of the pathological findings in 6 experimental goats after intratracheal inoculation

Goat No.	Age at inoculation (days)	Interval between inoculation and death (days)	Macroscopical pathology of the lung	Microscopical pathology of the lung	Remarks
1	2	145	No lesions evident	Not done	Died from coccidiosis
2	3	281	Miliary firm, grey-white, opaque, glistening nodules, varying in diameter from 2–7 mm present in all lobes	Multiple discrete foci of jaagsiekte present, as well as an interstitial pneumonia, with some perivascular and peribronchiolar lymphoid hyperplasia	Slaughtered <i>in extremis</i> as a result of acute meningitis
3	2	391	Nodules resembling those in Goat 2 present. Sizes vary between 1–10 mm. Most numerous in caudal lobes	Multiple foci of jaagsiekte resembling those in Goat 2. Mild interstitial pneumonia present in parts of lung	—
4	7	404	Single subpleural nodule 1.5 mm in diameter present, resembling those in Goat 2	Nodule noticed macroscopically comprising lymphoid hyperplasia	Negative for jaagsiekte
5	7	434	Single nodule, 2 mm in diameter present, resembling those in Goat 2	Fairly severe interstitial pneumonia present, with lymphoid hyperplasia and mild epithelialization. Nodule not examined histologically	Jaagsiekte not confirmed histologically
6	3	1 070	One irregular, sunken, consolidated, hyperaemic area, about 50 × 50 mm in extent, at junction of right cranial and middle lobes. Numerous firm, white foci, 1–3 mm in diameter, distributed through both lungs, especially subpleurally	Widespread, chronic, interstitial pneumonia, in parts accompanied by atelectasis. Scattered foci of lymphoid hyperplasia	Negative for jaagsiekte

TABLE 2 Immune blot results showing the cross reaction between the different goat sera and SA-lentivirus antigens before and after inoculation

Goat No.	Sera before inoculation ¹		Sera at slaughter ¹	
	p28	p16	p28	p16
1	NT	NT	NT	NT
2	—	—	NT	NT
3	—	—	—	—
4	—	—	+	F
5	—	—	+	—
6	—	—	+	+

¹ Sera were diluted 1/10

— = no detectable cross reaction

F = faint detectable band in the p28 or p16 region

+

NT = not tested

in the upper 2/3 of the caudal lobes and lower half of the middle lobes. Very few were present in the cranial lobes. The nodules could easily be detached or “shelled-out” from the normal surrounding lung tissue. The appearance of the lesions in Goats 2 and 5 very closely resembled those in the previous case. In Goat 2 they were relatively numerous, varied in diameter from 2–7 mm and were more or less evenly distributed throughout all lobes of the lungs (Fig. 1), while in Goat 5 only a single subpleural nodule about 2 mm in diameter was noticed.

In the lungs of Goat 4 a subpleural, grey-white nodule of lymphoid hyperplasia about 1.5 mm in diameter was observed. Goat 6, which was slaughtered in apparent good health 1070 days after inoculation, showed an irregularly shaped area at the junction of the right cranial and middle lobes, which was about 50 × 50 mm in extent and was sunken, red and of increased consistency. In addition, in this animal there were also fairly numerous small (1–3 mm in diameter) firm, dense, white gli-

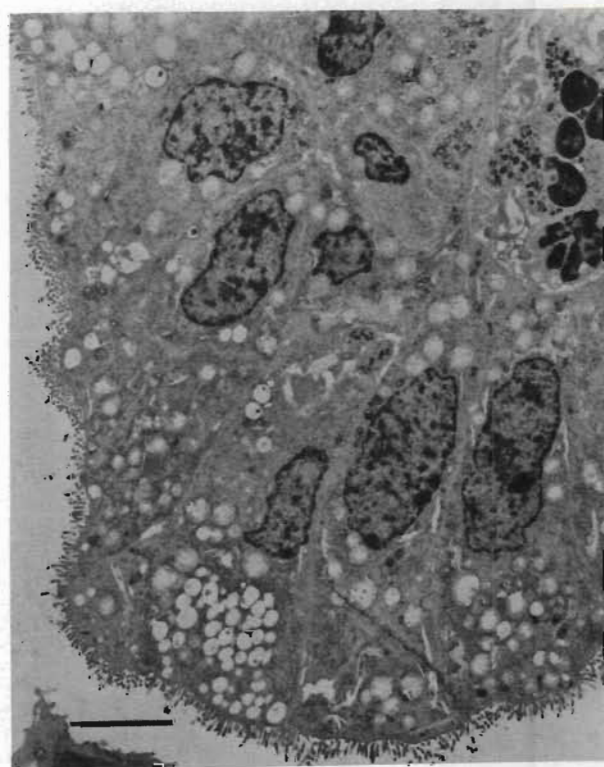


FIG. 10 Columnar tumour cells lining the alveolar lumen. Bar = 5 μm

tening foci of lymphoid hyperplasia scattered throughout the lung parenchyma, most, however, being located subpleurally.

Lesions of intestinal coccidiosis were present in Goat 1 which were responsible for the death of this animal.

No other pathological changes considered to be of significance were observed macroscopically in any of the animals.

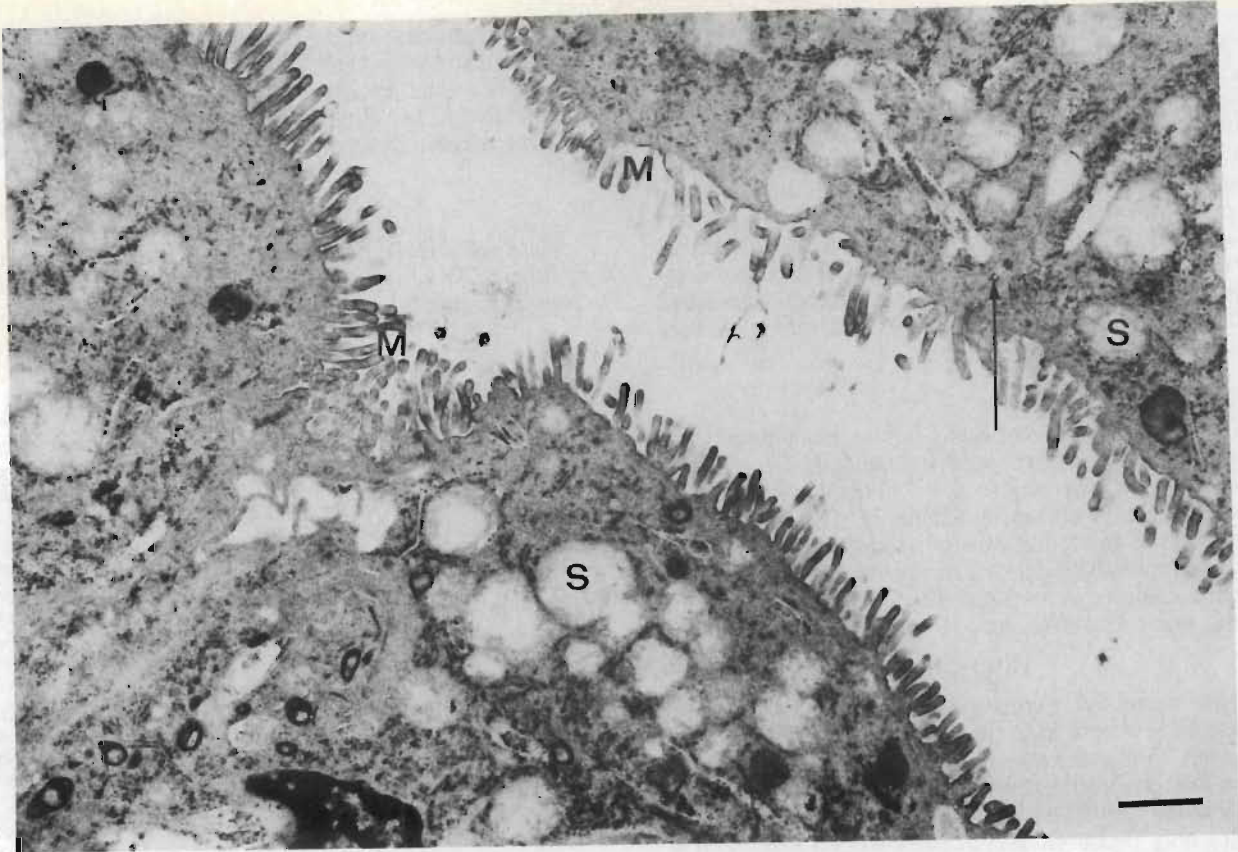


FIG. 11 Secretory granules (S) in tumour cells. Note the microvilli (M) on the surface of the tumour cells and junctional complexes (arrow). Bar = 1 μ m

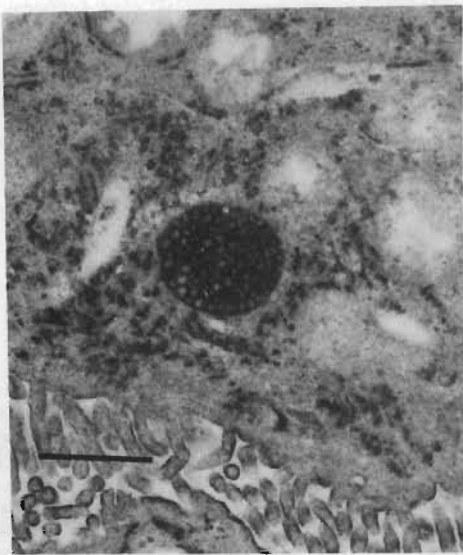


FIG. 12 Multivesicular body in a tumour cell. Bar = 1 μ m

Histologically the nodules seen macroscopically in Goats 2 and 3 consisted of lesions typical of those of ovine jaagsiekte or bronchiolo-alveolar adenocarcinoma (Fig. 2 & 3). The neoplastic epithelial cells were well differentiated, being mainly one layer thick, while the cords were separated from each other by relatively delicate fibrous connective tissue. In some of the lesions, mild perivascular and peribronchiolar lymphoid hyperplasia was present, and in most a neutrophil infiltration into the lumina formed by the tumour cells was quite pronounced (Fig. 4). The lesions were well circumscribed but were not encapsulated (Fig. 5).

In Goats 2, 3, 5 and 6 an interstitial pneumonia was noticed on microscopical examination. The lungs of Goat 1 were not examined histologically. The pneumonia varied in degree, being most pronounced in Goat 5 and least severe in Goat 3. It consisted primarily of a thickening of the alveolar walls caused by an infiltration particularly of macrophages but also of lymphocytes and mild fibrous connective tissue proliferation (Fig. 6 & 7). In areas where these changes were marked, not all lobules being equally affected, there was a reduction in the number and size of alveoli. In some alveoli evidence of partial "epithelialisation" or hyperplasia of type II epithelial cells was observed, but this was not a striking phenomenon (Fig. 8). Perivascular, peribronchial and peribronchiolar lymphoid hyperplasia was present but not always very obvious except in Goat 6 where it was very marked and accounted for the numerous white foci seen macroscopically (Fig. 9). Exudation or infiltration of cells into the alveoli or bronchial lumina was not a feature of the lesion.

The single nodule encountered in the lungs of Goat 4 proved to be one of lymphoid hyperplasia. No other lesions were noticed on microscopical examination of the lungs of this animal.

Electron microscopy

The lesions found in Goat 3 were examined with TEM. Columnar tumour cells lined the alveolar lumen and in numerous areas papillae formation was observed (Fig. 10). The nuclei were usually situated towards the base of the cell and were regular in shape. The tumour cells in the lesion were epithelial in character, and resembled type II pneumocytes. There were well-defined junctional complexes connecting the cells and the apical surfaces of the tumour cells were covered with abundant

microvilli. Most tumour cells contained pleomorphic secretory granules (Fig. 11) which were usually located in the apical region of the cells. There were also smaller granules along the basal lamina. The granules varied from electron-dense to electron-lucent, but no myelinoid bodies were observed. Multivesicular bodies were present in many of the tumour cells (Fig. 12) and glycogen also in limited quantities. Mitochondria were fragile and ill-defined. This is possibly a preparation artifact, as cytoplasmic clefts were also observed. The alveolar lumina were mostly clear but in rare areas they were filled with electron-dense secretions. No viral particles were observed in the sections.

Serology

None of the sera collected before inoculation contained antibodies against lentiviral antigens. Seroconversion, however, was observed in 3 of the 4 animals tested at the time of slaughtering (Table 2). The highest concentration of antibodies was found in Goat 5, which also had the most severe interstitial pneumonia. No seroconversion could be demonstrated in Goat 3, which had the most pronounced JS lesions.

DISCUSSION

The successful experimental transmission of jaagsiekte to new-born kids clearly proves the susceptibility of goats to this disease. However, the fact that a viral dose that produced extensive lesions in more than 90 % of new-born lambs after 2–3 months (Verwoerd *et al.*, 1985) only produced small circumscribed lesions in at least 2 out of 5 goats after 9–13 months suggests that goats are much less susceptible than sheep. This is supported not only by the results of Sharp *et al.* (1986), which indicated that transmission of jaagsiekte to goats is less efficient and much slower than is the case in sheep, but also by previous reports of a very low incidence of the natural disease in goats. For example, 3 out of 1 410 goats slaughtered in Greece (Stefanou *et al.*, 1975) and 18 out of 3 956 goats in India (Rajya & Singh, 1964) had a low incidence of the disease. It may also be significant that the 2 goats that developed jaagsiekte lesions were inoculated 2 and 3 days after birth, respectively, whereas 2 of the 3 that did not develop lesions were inoculated only at the age of 7 days. It was found in sheep that the efficiency of transmission rapidly diminishes after birth (Verwoerd *et al.*, 1985). The nodular character of the lesions in the experimental disease in the goat described by Sharp *et al.* (1986) is similar to that present in the cases reported here.

The presence of lentivirus in the inoculum did not seem to influence the development of lesions, since no evidence of seroconversion and only very mild interstitial pneumonia was found in Goat 3, the animal which had the most pronounced jaagsiekte lesions. The relationship, if any, between these 2 sheep retroviruses which are so commonly found together still needs clarification.

The fact that jaagsiekte retrovirus infection in goats results in the development of a neoplasm closely resembling that caused by the same virus in sheep, that the same (or very similar) disease occurs naturally in goats

in some countries and the tumour is a bronchiolo-alveolar adenocarcinoma (Stünzi, Head & Nielsen, 1974) and not an adenoma lend support to the contention that the disease should be called jaagsiekte rather than ovine pulmonary adenomatosis or ovine pulmonary carcinomatosis.

ACKNOWLEDGEMENT

The help of Dr Tim O'Neill, Armed Forces Institute of Pathology, Washington DC, USA, with some of the necropsies is gratefully acknowledged.

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Characteristics of a Novel Lentivirus Derived from South African Sheep with Pulmonary Adenocarcinoma (Jaagsiekte)

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A novel lentivirus was isolated from South African sheep with experimentally transmitted lung adenocarcinoma. Similar to visna virus and caprine arthritis encephalitis virus, this new strain induced cytopathic effects on ovine plexus choroid cultures. In contrast to a recent Israeli isolate from sheep with adenocarcinoma, the South African lentivirus could not transform fibroblast cultures. The antigenic relatedness between the new isolate and visna virus was assessed by immunoprecipitation of radiolabeled viral proteins, using monospecific antisera against visna virus proteins. The results indicate that the new virus contains four major structural proteins of sizes similar to those of visna virus (i.e., gp135, p30, p16, and p14) and have some common antigenic determinants (about 90% in the major core antigen p30). However, the nucleotide sequences of the novel lentivirus were found to be only 16.5 to 27.4% homologous to visna virus and 8.3 to 15% homologous to caprine arthritis encephalitis virus, by means of liquid hybridization under stringent conditions. The genetic divergence indicated by this last result was confirmed by the dissimilar restriction endonuclease cleavage map of the new virus in comparison to those of visna virus and three caprine arthritis encephalitis virus strains. The demonstration of a third type of ovine lentivirus supports the concept of an important genetic variation among the lentiviruses infecting one animal species. © 1987 Academic Press, Inc.

INTRODUCTION

Sheep pulmonary adenocarcinoma, or jaagsiekte, is a contagious lung cancer which is characterized, inter alia, by a copious secretion that accumulates in the respiratory tract (jaagsiekte lung fluids) (Verwoerd *et al.*, 1985). The tumor can be transmitted experimentally by intratracheal or intrapulmonary inoculation of newborn lambs with tumor homogenates or lung fluids.

The detection of retroviruslike particles and reverse transcriptase activity in those preparations and in experimentally induced tumor tissue suggested strongly the involvement of a retrovirus (Martin *et al.*, 1976; Verwoerd *et al.*, 1980; Sharp *et al.*, 1983). Recently an immunological study by Western blotting of the antigens present in tumors and lung fluids revealed that this retrovirus contains a 25,000 mol wt polypeptide which cross-reacts antigenically with the 27,000 mol wt core protein (p27) of Mason-Pfizer monkey virus (MPMV) but not with the p30 of the lentiviruses, the prototypes of which are visna virus and caprine arthritis encephalitis virus (CAEV) (Sharp and Herring, 1983).

By concentrating and partially purifying the virus present in the lung fluids, several groups have been able to dramatically reduce the incubation period and to induce tumors in a few weeks (Verwoerd *et al.*, 1980;

Sharp *et al.*, 1983), suggesting strongly that the MPMV-related retrovirus has an etiological role in jaagsiekte. However, a group in Israel has reported the isolation from a jaagsiekte tumor of a retrovirus with type C morphology that shows 30% homology by DNA-RNA hybridization with various lentiviruses (Irving *et al.*, 1984) and that possesses some transforming and oncogenic properties (Safran *et al.*, 1985). In this paper we report the isolation and a preliminary characterization of a lentivirus from a jaagsiekte tumor cell line. This novel isolate is genetically distinct from visna virus and CAEV. However, in contrast to the Israeli lentivirus, it does not exhibit transforming properties in tissue culture and induces cytopathic effects such as visna virus and CAEV.

MATERIAL AND METHODS

Viruses

The prototype strain of visna virus is the Icelandic strain K1514. Three strains of CAEV were grown for analysis, namely, USA-CAEV-1 (C0) isolated by Cork *et al.* (1974), USA-CAEV-2 (75 G63) isolated by Crawford *et al.* (1980), and F-CAEV-1 (RU) isolated by Russo (1982). South African lentiviruses were obtained from tumor cell line 15.4 generated from sheep experimentally infected with jaagsiekte lung fluids and affected with lung adenocarcinoma (Coetzee *et al.*, 1976). Ovine

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RESULTS

Isolation of lentiviruses from South African sheep with experimentally transmitted jaagsiekte

The original discovery of a South African lentivirus was made by chance during attempts to cultivate the MPMV-related jaagsiekte retrovirus (JSRV) present in lung fluids (Verwoerd *et al.*, 1983, 1985). A lung rinse pellet, which was obtained from an experimental case of jaagsiekte, as previously described (Verwoerd *et al.*, 1980), and which contained reverse transcriptase activity, was used to infect ovine choroid plexus (OCP) cells. After a latent period of several weeks, the presence of a lentivirus was detected by the appearance of cytopathic effects typical of lentiviruses, i.e., formation of fused giant cells and refractile dying cells. This first isolate, which was cloned with endpoint dilution, was found to be less lytic than visna virus, but unlike the persistent ovine lentiviruses (Quérat *et al.*, 1984), it was unable to maintain persistent infections on passaging the cell cultures. It was consequently called South African ovine maedi-visna virus, isolate 1 (SA-OMVV-1) (Payne *et al.*, 1986). The discovery of SA-OMVV-1 was confirmed in France by isolating another lentivirus directly from the tumor cell line 15.4 (SA-OMVV-2). This cell line was established in 1975 from a jaagsiekte case and formed the basis of the jaagsiekte transmission studies. After endpoint dilution cloning and several treatments against mycoplasma, this clonal isolate was found to cause the formation of very large syncytia of OCP cells (Fig. 1). These cells cannot be passaged as chronically infected cells. This strong tendency of SA-OMVV-2 toward cell fusion is similar to that of the Israeli lentivirus, recently isolated from sheep with pulmonary adenocarcinoma (Safran *et al.*, 1985). However, in contrast to the latter virus, SA-OMVV does not possess transforming properties such as foci formation or colony growth in soft agar by various ovine or caprine cell cultures (data not shown).

Nucleic acid homology between SA-OMVV-2 and the two lentivirus prototypes, visna virus and CAEV

We have previously shown that ovine lentiviruses can belong to two types. Ovine maedi-visna viruses (OMVV) type I are highly lytic and closely related to visna virus. OMVV type II, which are persistent in ovine cells, are closely related to CAEV. To determine the nucleic acid homology between SA-OMVV to type I and type II lentiviruses, we performed liquid-phase hybridization among the RNA genomes of five lentiviruses, namely, visna virus, three strains of CAEV and SA-OMVV-2, and the complementary single-strand DNA of visna virus, USA-CAEV-2 (75G63 strain), and SA-OMVV-2. Ge-

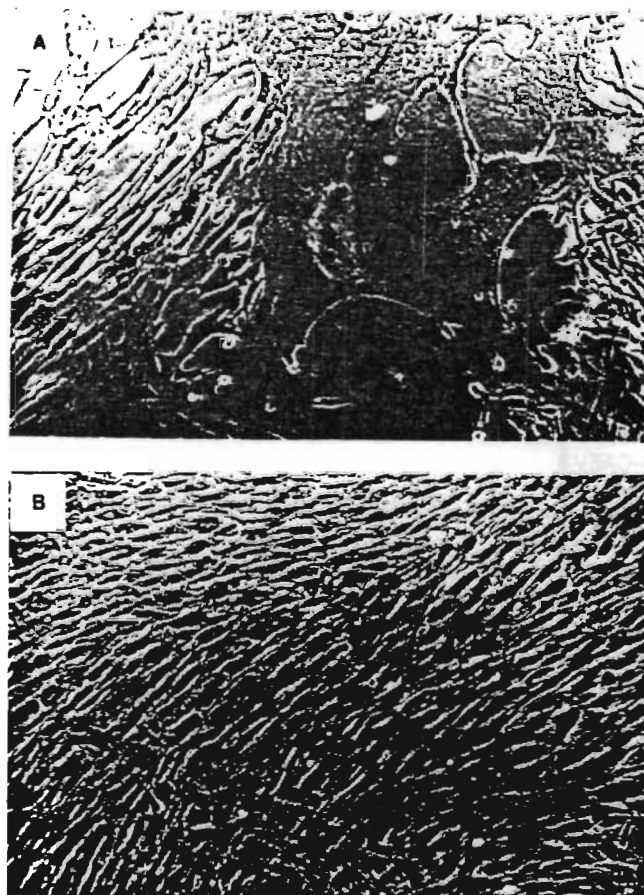


Fig. 1. Morphological characteristics of an ovine cell culture infected by the novel lentivirus isolated from South African sheep with jaagsiekte. Ovine fibroblasts infected with endpoint diluted SA-OMVV-2 (A) and normal ovine fibroblasts F-OCP₁₀ (B) photographed with phase-contrast optics.

nomic RNAs were extracted from extracellular virions, and poly(A)-containing RNA was isolated by means of an oligo(dT)-column. ³²P-labeled cDNAs were obtained by reverse transcription of poly(A)-containing virion RNA in the presence of actinomycin D and DNase-digested calf thymus DNA primer (Quérat *et al.*, 1984). Table 1 shows that the extent of cross-hybridization between visna virus and SA-OMVV-2 ranges from 16.5 to 27.4%, and that between USA-CAEV-2 and SA-OMVV-2 from 8.3 to 15%. It is therefore clear that SA-OMVV-2 is distinct from visna virus and CAEV and represents a novel type of lentivirus.

In contrast, the three strains of CAEV tested are largely homologous, hybridizing to the extent of 86 and 88.1%, respectively, for F-CAEV-1 (Russo strain) and USA-CAEV-1 (Cork strain) relative to USA-CAEV-2 (75G63 strain).

Restriction map of SA-OMVV-2

Since the nucleic acid homologies among SA-OMVV-2, visna virus, and CAEV are low, the restriction map

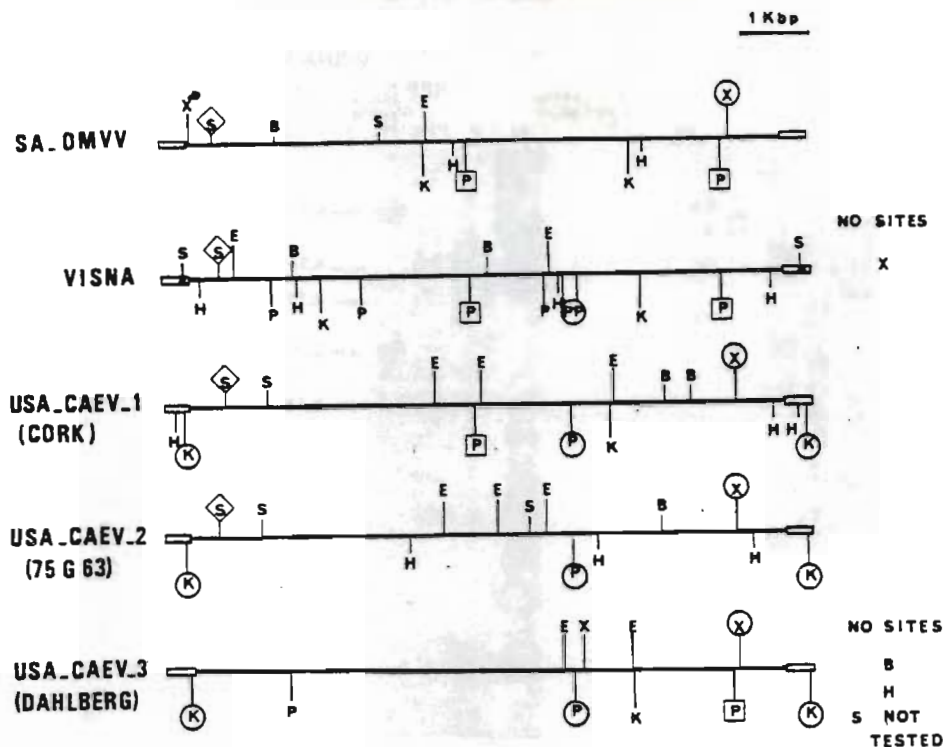


FIG. 4. Comparison of the restriction enzyme map of SA-OMVV DNA with those of visna virus and three strains of CAEV. The comparison of the physical maps was limited to seven restriction enzymes: *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Kpn*I (K), *Pst*I (P), *Sst*I (S), and *Xho*I (X). The map of visna virus was derived from the published nucleotide sequence (Sonigo *et al.*, 1985). Those of USA-CAEV-1 (Cork strain) and USA-CAEV-2 (strain 75G63) were reconstructed in the laboratory and were similar to those published (Pyper *et al.*, 1984; Roberson and Cheevers, 1984), except for USA-CAEV-2, where three sites (2 *Bam*HI and 1 *Hind*III) were presently absent. The map of USA-CAEV-3 is according to that recently reported (Yaniv *et al.*, 1985). All the CAEV proviruses were adjusted to a length of 9.6 kbp, which is likely common to all the lentiviruses. The site S enclosed by a diamond is common to all the lentiviruses. Symbols enclosed within a circle are common to all three CAEV strains (Yaniv *et al.*, 1985); those enclosed by a square are common to visna virus and SA-OMVV-2.

tracellular virions in comparison with those of visna virus, USA-CAEV-1 (Cork strain), and F-OMVV-1 (II) (Qu  rat *et al.*, 1984). Figure 5 indicates that SA-OMVV-2 (III) contains structural proteins with sizes similar to that of visna virus, i.e., three internal proteins p30, p16, and p14 and one major glycoprotein gp135 (respectively, lanes A and B). In contrast, visna virus proteins are quite distinct in molecular weight from CAEV and type II OMVV, which contain a gp120 and three internal proteins p30, p18, and p14.5. Therefore, type III OMVV cannot be distinguished from visna virus with regard to the size of the structural proteins.

To further characterize the antigenic relationship between the proteins of SA-OMVV (III) and visna virus, we performed a comparative analysis of viral proteins during their synthesis in cells infected with both virus types instead of working with extracellular virions which are poorly produced in the case of SA-OMVV (III). Briefly, infected cells were labeled with [³⁵S]methionine, and the extracted viral proteins were immunoprecipitated with three monospecific sera raised against the two internal proteins (p30 and p16) and the major envelope glycoprotein (gp135) of the Icelandic strain

K1514 of visna virus. Figure 6 shows the results of these experiments. When proteins from cells infected with type III lentivirus were immunoprecipitated with visna virus anti-p30 and anti-p16 sera, both mature p30 and p16 of SA-OMVV (III) were recognized as well as precursor intermediates Pr55^{gag} and p50^{gag}. Therefore, SA-OMVV (III) and visna virus share some common antigenic determinants on both proteins p30 and p16, while CAEV and visna virus could be distinguished by an absence of cross-immunoreactivity among their proteins smaller than the p30s (lane II AB). An investigation of *env*-related antigens present in cells infected with SA-OMVV (III) was also performed using both visna virus anti-gp135 serum and SA-OMVV (III) anti-gp135 serum. Both sera reacted similarly with gp135-related antigens in visna virus-infected cells (lanes C I and D I) and revealed a major *env*-product, the precursor gPr150^{env} (Vigne *et al.*, 1982). In cells infected with SA-OMVV (III) (lanes C III and D III), the *env*-related precursors were expressed at a very low level. Mature gp135 of visna virus and SA-OMVV were present in very low amounts in their respective infected cells.

Last, the antigenic relatedness of SA-OMVV-2 p30

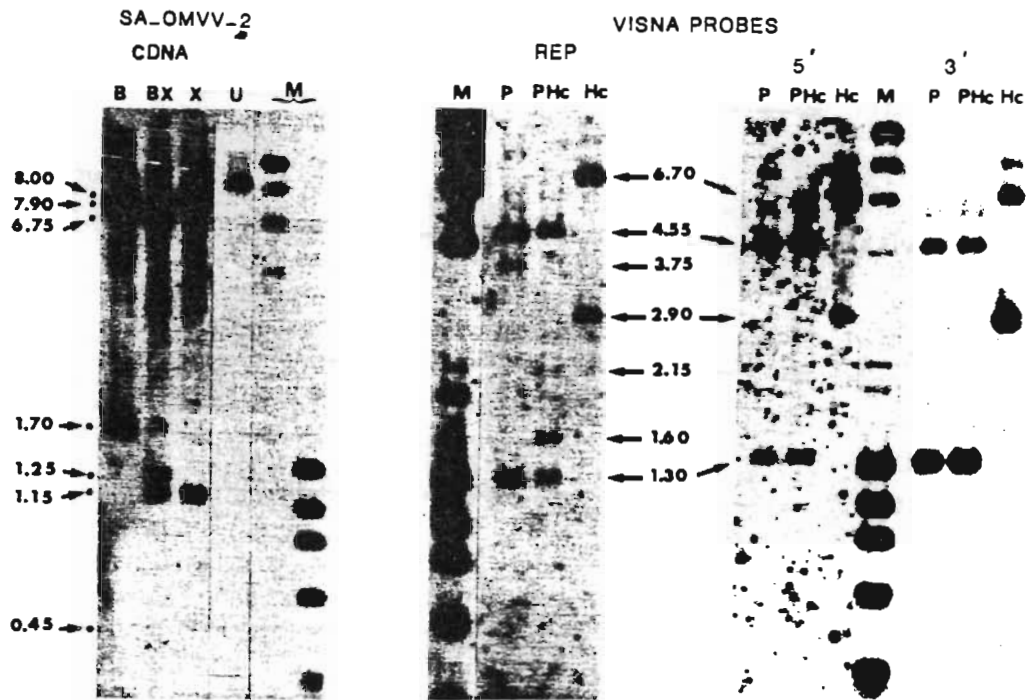


FIG. 2. Proviral DNA analysis of SA-OMVV-2. F-OCPI₀ cells were infected with SA-OMVV-2. Unintegrated proviral DNAs were extracted from the infected cells by the Hirt procedure and digested by *Bam*HI (B), *Xho*I (X), *Pst*I (P), *Hinc*II (Hc), and combinations of them as indicated by symbols above the lanes. Lane U represents undigested SA-OMVV-2 DNA. Lanes M are *Hind*III-digested fragments of λ phage DNA and *Hae*III-digested fragments of ϕ X phage DNA. The digested and undigested DNAs were analyzed on a 1% agarose gel, transferred to nitrocellulose, and hybridized independently with [³²P]cDNA representative of the whole genome of type III lentivirus (panel SA-OMVV-2 cDNA) and with ³²P nick-translated DNAs of visna virus representative of its whole genome of visna virus, of K1514 (panel Rep visna), of its 5' unique sequences (panel 5') or of its 3' unique sequences (panel 3'). Sizes (in kbp) of the viral DNA fragments are indicated on the sides of the panels. The 0.45-kbp fragment was visible in lanes X and BX (panel SA-OMVV-2 cDNA) of the original autoradiogram.

pared (Yaniv *et al.*, 1985), the Cork strain (Pyper *et al.*, 1984), the 75G63 strain (Roberson and Cheevers, 1984), and the Dahlberg strain (Yaniv *et al.*, 1985). Figure 4 shows that the restriction map of SA-OMVV is largely distinct from that of visna virus and those of CAEV strains. Nevertheless, it is noteworthy that some sites are shared by SA-OMVV and other lentiviruses, such as the *Sst*I site at position ca. 1 kbp and the *Xho*I site common to all the three CAEV at position 8.85 kbp. In conclusion, the present data confirm that SA-

OMVV-2 is the prototype of a new type of ovine lentivirus, henceforth designated SA-OMVV type III (SA-OMVV (III)).

Protein analysis and antigenic relationship among the structural proteins of the three types of lentiviruses

To identify the structural proteins of SA-OMVV-2 (III) we analyzed the ³⁵S-labeled methionine proteins of ex-

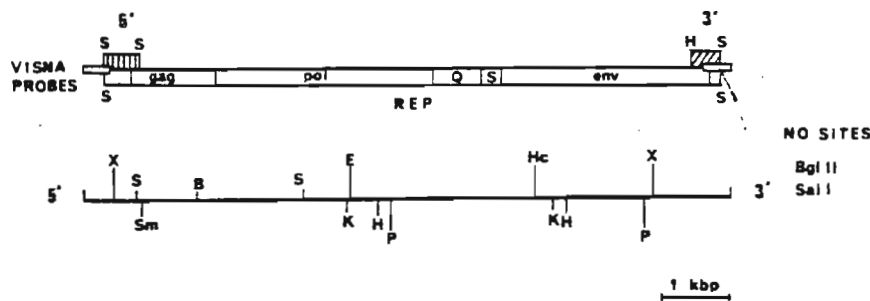


FIG. 3. Restriction enzyme map of SA-OMVV-2 proviral DNA. Unintegrated proviral DNA of SA-OMVV-2 was mapped with nine restriction enzymes: *Bam*HI (B), *Eco*RI (E), *Hinc*II (Hc), *Hind*III (H), *Kpn*I (K), *Pst*I (P), *Sma*I (Sm), *Sst*I (S), and *Xho*I (X). Orientation of the genome was established by hybridization analysis of digested SA-OMVV-2 DNA with 5' and 3' probes of visna virus. The portions of visna virus genome that they represent are shown in the upper half of the map of SA-OMVV-2.



Fig. 7. Relatedness of the p30 antigens of SA-OMVV-2 and visna virus. SA-OMVV-2 and visna virions, purified as previously described (Vigne *et al.*, 1977), were disrupted by 1 hr of incubation in lysis buffer and then tested in a large excess of core virion antigens (20 μ g) for their ability to compete with [35 S]methionine-labeled visna virus proteins. Visna virus anti-p30 serum (0.5 μ l) and unlabeled antigens were incubated 1 hr at room temperature and overnight at 4°, and then [35 S]methionine-labeled intracellular visna viral proteins were added in a final volume of 500 μ l. After incubation for 4 hr at 4°, antigen-antibody complexes were precipitated by the addition of 50 μ l of *Staphylococcus aureus* (10%) and centrifugation at 10,000 g for 1 min. Immunocomplexes were then dissociated from the bacteria and analyzed in a sodium dodecyl sulfate-6 to 18% polyacrylamide gel as previously described (Vigne *et al.*, 1982). Lane A represents p30-related proteins of visna virus-infected cells directly immunoprecipitated by the visna virus anti-p30 serum. Lanes B and C, similar assays with visna virus anti-p30 serum preadsorbed to 20 μ g of purified SA-OMVV and visna virus, respectively. The counts per minute present in the p30 protein bands cut from the gel after autoradiography in lanes A, B, and C allowed us to determine that the percentages of binding in the presence of competing SA-OMVV and visna virus antigens were 11.3 and 0%, or 100% in the absence of competing antigens.

netic and antigenic relationship with SA-OMVV (III) is not known.

SA-OMVV (III) was clearly distinguished from visna virus and CAEV by analyzing their nucleic acid homologies by means of liquid hybridization or on Southern blots. The 16.5 to 27.4% homology between SA-OMVV (III) and visna virus, compared to only 8.3 to 15% with CAEV, suggests that SA-OMVV (III) is more closely related to visna virus than to CAEV. It is noteworthy that the extent of hybridization between visna virus and CAEV reported here is lower than that previously published (Roberson *et al.*, 1982; Gazit *et al.*, 1983). This difference can be explained by the fact that our cDNAs were synthesized from highly purified poly(A)-containing

RNA in a primed reaction with avian myeloblastosis virus reverse transcriptase and not in an endogenous reaction from viral particles which may contain packaged cellular RNA.

Southern blotting analyses of SA-OMVV-2 proviral DNA hybridized under low stringency conditions with Rep visna probe suggested that the putative LTRs and *gag* and *pol* regions of SA-OMVV-2 are probably more homologous to the corresponding regions of visna virus than are the putative *env* region (Fig. 2). These preliminary observations correlate well with a previous study on nucleotide sequence homologies between CAEV and visna virus DNAs (Pyper *et al.*, 1984, 1986).

The demonstration that SA-OMVV (III) is related more closely to visna virus than to CAEV was confirmed at the protein level. Visna virus and SA-OMVV (III) possess proteins of similar sizes (gp135, p30, p16, and p14) and share some common antigenic determinants at least in their gp135, p30, and p16 (90% of homology in their major core protein p30). CAEV, in contrast, contains some proteins with distinct sizes, namely, gp120, p30, p18, and p14.5, and more unshared antigenic determinants, particularly in the small proteins p18 and p14.5, since the visna virus anti-p16 serum could not immunoprecipitate *gag*-related antigens in CAEV-infected cells (Quérat *et al.*, 1984). Nevertheless, differences in the level of expression of the *env*-related polypeptides of SA-OMVV (III) and visna virus should be pointed out. Whereas visna virus-infected cells contain high amounts of polypeptide precursor to gp135 (Pr150^{env}), SA-OMVV (III)-infected cells do not. This distinction in the *env*-precursor expression was also true for visna virus relative to CAEV. This might indicate a relationship between the high lytic properties of Icelandic visna virus and the very high production of *env*-precursors in the infected cells.

Analysis of the biological and biochemical properties of the novel lentivirus revealed that the only accurate diagnostic test for its identification was the Southern blot analysis of its proviral DNA, which showed a restriction pattern quite distinct from those of visna virus and CAEV. It will be interesting therefore to characterize the various strains of lentiviruses previously isolated in the world, but never biochemically identified, to examine the eventual presence of other type III isolates. Indeed, if the endemic spread of jaagsiekte is correlated to the presence of type III lentivirus, this novel virus type should be present in numerous countries in the world, since jaagsiekte is distributed worldwide (Verwoerd *et al.*, 1985).

The pathogenic role of type III OMVV in the induction of lung neoplasia is not known; it is, however, noteworthy that experimental transmissions of jaagsiekte

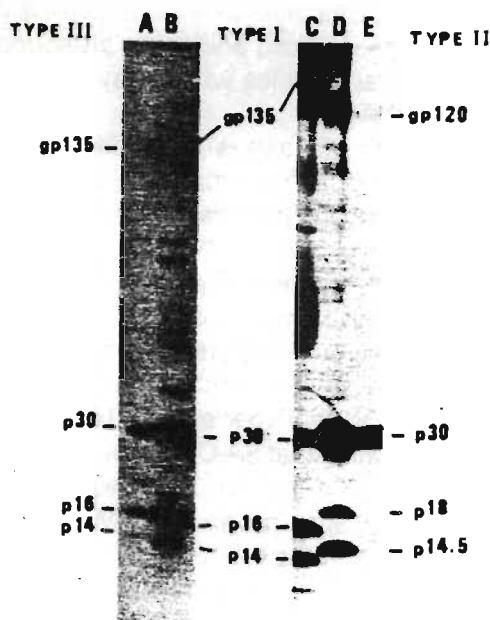


FIG. 5. The polypeptide pattern of the novel lentivirus compared to those of visna virus (or OMVV (I)) and USA-CAEV-1 (or OMVV (II)). Extracellular particles of visna virus, K1514 (lanes B and C), and SA-OMVV-2 (lane A) were produced in F-OCF₁₀; those of USA-CAEV-1 (lane D) and F-OMVV (II) (lane E) were produced in F-CFSM₁ cells. Purified particles were disrupted by SDS, and viral proteins were analyzed into SDS-6 to 18% polyacrylamide gel. The sizes (in kilodaltons) of the major proteins of the three types of lentiviruses are indicated.

to that of visna virus was measured by competing the binding of visna virus anti-p30 serum to [³⁵S]-methionine-labeled visna virus p30 antigen with purified SA-OMVV-2 and visna virions. Figure 7 indicates that a large excess of SA-OMVV virion antigens (lane B) could not fully inhibit the binding of visna virus p30 to the visna virus anti-p30 serum, whereas similar amounts of visna virion antigens completely competed in a homologous system (lane C). Therefore, the p30 antigens of both types of lentiviruses contain some distinct determinants (ca. 10%). A similar result was obtained with p16 antigens (data not shown). This kind of assay was, however, not presently possible for comparing the gp135-related antigens, since the envelopes of SA-OMVV virions were systematically lost at the first step of the purifications.

DISCUSSION

The South African isolate SA-OMVV-2, which has been characterized in this paper, represents a novel type of ovine lentivirus which is genetically distinct from the two former types of lentiviruses, the prototypes of which are visna virus (type I) and CAEV (type II). The type III lentivirus is interesting in that it has been isolated

from sheep with pulmonary adenocarcinoma (jaagsiekte), whereas types I and II were associated with inflammatory diseases without neoplastic involvement, i.e., demyelinating encephalitis (visna), interstitial pneumonia (maedi or progressive pneumonia), or chronic arthritis (arthritis-encephalitis syndrome) (Sigurdsson *et al.*, 1952, 1957; Thormar and Paisson, 1967; Cork *et al.*, 1974; Crawford *et al.*, 1980). However, SA-OMVV-2, which was directly isolated from an established pulmonary adenocarcinoma cell line (Coetzee *et al.*, 1976), does not transform the infected OCP cells but shares with all the other lentiviruses lytic properties, including syncytia formation. These observations on SA-OMVV (III) are different from those of Perk and collaborators who recently isolated a novel lentivirus with several transforming properties from the lungs of an Israeli case of lung adenocarcinoma (Irving *et al.*, 1984; Safran *et al.*, 1985). Since this retrovirus has not yet been biochemically characterized, its ge-

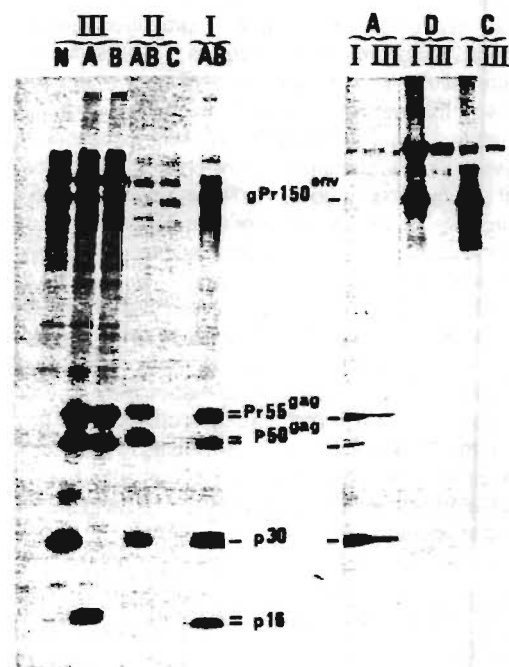


FIG. 6. Type III lentiviruses share antigenic determinants with visna virus in both p30 and p16 internal proteins. F-OCF₁₀ or F-CFSM₁ cells infected by various lentiviruses were labeled for 4 hr with [³⁵S]methionine. Intracellular viral proteins of SA-OMVV-2 (III) in ovine cells (lane III), of USA-CAEV-1 in caprine cells (lane II), and of visna virus in ovine cells (lane I) were analyzed by immunoprecipitation with three monospecific sera to three major proteins of visna virus K1514, namely, p30 (lane A), p16 (lane B), p30 and p16 (lanes AB), and gp135 (lane C). Lane D represents lysate proteins of visna virus (lane I)- or SA-OMVV-2 (lane III)-infected ovine cells immunoprecipitated by an anti-SA-OMVV sheep serum, which is largely anti-gp135, since only a large polypeptide of 150,000 Da is immunoprecipitated in visna virus-infected cells, as is the case with a monospecific anti-gp135 serum shown in lane C. Lane N shows nonspecific precipitations of proteins by normal sheep serum.

were successfully performed in South Africa with extracts from a tumor cell line which contained the SA-OMVV lentivirus. Our inability to transform fibroblast cultures with SA-OMVV-2 in focus or colony assays (data not shown) suggested that this novel lentivirus is not intrinsically oncogenic. Therefore it is possible that the SA-OMVV lentivirus could rather act as a cofactor in the induction of jaagsiekte by JSRV.

In preliminary experimental infections of sheep and goats injected intratracheally, SA-OMVV did not induce any symptoms within 2 years. When the virus was inoculated into the joints, however, it provoked a transient arthritis (Payne *et al.*, 1986). In contrast, neoplasmas could be induced within a few weeks when sheep were inoculated intratracheally with partially purified JSRV (Verwoerd *et al.*, 1980; Sharp *et al.*, 1983).

However, since this retrovirus has not yet been successfully passaged in cell cultures and has not been biologically cloned (Verwoerd *et al.*, 1983), its precise etiological role in neoplasm formation cannot be defined. Further virological studies will be necessary to determine the respective roles of the two types of retroviruses in the pathology of jaagsiekte.

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ISOLATION AND PRELIMINARY CHARACTERIZATION OF THE JAAGSIEKTE RETROVIRUS (JSRV)

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ABSTRACT

VERWOERD, D. W., PAYNE, ANNA-LISE, YORK, D. F. & MYER, M. S., 1983. Isolation and preliminary characterization of the jaagsiekte retrovirus (JSRV). *Onderstepoort Journal of Veterinary Research*, 50, 309-316 (1983)

Jaagsiekte, or ovine pulmonary adenomatosis, is caused by a recently discovered retrovirus. The virus cannot be cultivated *in vitro* at present, but a procedure is described for the isolation and purification of small amounts in the form of immune complexes with IgA from affected lungs. The virion was shown to possess a 70S RNA genome which can be transcribed by an endogenous reverse transcriptase. Nine polypeptides, ranging in size from 94 000 to 25 000 daltons, were found in purified preparations. Using neutralization of the viral reverse transcriptase and an enzyme immunoassay as criteria, no serological relationship could be demonstrated to representatives of type B, C and C oncoviruses, or to bovine leukemia virus, maedi-visna virus of sheep or caprine arthritis-encephalitis virus.

INTRODUCTION

Jaagsiekte, or ovine pulmonary adenomatosis, was first described as a disease entity in South Africa in the early nineteenth century but was later found to be almost world-wide in its distribution. It was known to be an infectious disease long before its neoplastic nature was appreciated (Tustin, 1969). Many years of scientific investigation in various countries failed to reveal the aetiological agent, although a variety of organisms was isolated from affected lungs and incriminated as possible causal agents (Wandera, 1971).

A viral aetiology has long been suspected as a result of filtration experiments, and an ovine herpesvirus was the first candidate to be isolated by various groups. Transmission experiments with this virus were unsuccessful, however (De Villiers & Verwoerd, 1980).

The possible involvement of a retrovirus was first suggested by the observation of particles possessing typical type C retrovirus morphology in sections of adenomatous lungs (Perk, Hod & Nobel, 1971). This was followed by the demonstration of retroviruses in cell cultures established from affected lungs (Malmquist, Krauss, Moulton & Wandera, 1972) and biochemical evidence for the presence of reverse transcriptase activity in lung extracts (Perk, Michalides, Spiegelman & Schlom, 1974). None of these studies included transmission experiments or excluded the possibility that the viruses observed were maedi-visna virus, however. Convincing evidence that a retrovirus is indeed the aetiological agent of the disease has only recently been produced (Verwoerd, Williamson & De Villiers, 1980; Herring, Sharp, Scott & Angus, 1983). This evidence consisted mainly of repeated serial transmissions and the demonstration of an inverse relationship between viral concentration and the incubation period of the disease. Jaagsiekte is usually classified as one of the 'slow' viral diseases, with an incubation period measured in years. By concentrating and partially purifying the virus we have been able to reduce this lag phase to weeks (Verwoerd *et al.*, 1980).

Lack of an *in vitro* system to cultivate the virus has so far precluded any biochemical study of the virion itself. In this paper we report on the isolation and purification of sufficient quantities of virus for a preliminary characterization.

MATERIALS AND METHODS

Abbreviations used: JSRV, jaagsiekte retrovirus; MMTV, mouse mammary tumor virus; MuSV, murine sarcoma virus; SMRV, squirrel monkey retrovirus; BLV, bovine leukemia virus; CAEV, caprine arthritis encephalitis virus; RDP, RNA-dependent DNA polymerase (reverse transcriptase); DTT, dithiothreitol; SDS, sodium dodecyl sulphate; ELISA, enzyme linked immunosorbent assay; MPMV, Mason-Pfizer monkey virus; MVV, maedi-visna virus.

Source of viruses and cell cultures

JSRV was isolated from a field case of jaagsiekte and serially passaged in sheep. The MMTV (strain R III) producing R III-MT cell line and the SMRV producing foetal canine thymus cell line (FCf2Th) were obtained from the American Type Culture Collection and grown according to their recommendations. MuSV was produced by the M(52)B cell line derived from a MuSV-induced mouse sarcoma at the National Cancer Institute, NIH, Bethesda. BLV was produced in a bat cell line, Bat₂-Cl₁, obtained from Dr J. Ferrer of the Bovine Leukemia Research Unit, University of Pennsylvania, New Bolton Center.

Purification of viruses

All the retroviruses produced in cell cultures were purified in the same way. One-and-a-half litre batches of cell culture medium were clarified by low speed centrifugation (30 min at 2 000 rpm) and then pelleted in a Beckman Ti-15 batch rotor for 2 h at 30 000 rpm. All procedures were carried out at 4 °C. Pelleted virus was suspended in buffer B (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 0.5 mM EDTA, 0.1 M KCl and 1 mM DTT), and stored at -70 °C until used. Preliminary purification was obtained by zonal centrifugation through a 5-30 % sucrose gradient in buffer B for 60 min at 30 000 rpm (SW41 rotor). The visible viral band was then layered on a 20-55 % sucrose gradient in buffer B and centrifuged for 16 h at 25 000 rpm (SW27 rotor) for final isopycnic purification. The 1.16-1.18 density fractions were collected and the virus pelleted for 90 min at 30 000 rpm (rotor 30). Pellets were suspended in buffer B for immediate use or storage at -70 °C.

Production and purification of JSRV

JSRV was produced in new-born lambs by injecting semi-purified virus, obtained from previous lung washes, intratracheally. Inoculation of 1×10^6 RDP

units (see below) produced clinical symptoms (dyspnoea) in 6–12 weeks in the majority of animals. When terminally ill, the lambs were slaughtered, the lungs were removed and immediately rinsed with 1,5 l of ice-cold PBS or cell culture medium. All further steps were carried out at 4 °C. The lungs were rinsed by pouring 500 ml of medium into the trachea, massaging the lungs well and pouring out the rinse fluid. This was repeated 3–4 times. The rinse fluid was then clarified by low speed centrifugation (30 min at 3 000 rpm) to remove cells and cell debris and then pelleted in a Ti 15 batch rotor for 2,5 h at 30 000 rpm. The pelleted material was scraped off the rotor wall with a rubber policeman, suspended in 0,05 M phosphate buffer (pH 7,4) containing 0,5 mM EDTA and 1 mM DTT and stored at –70 °C.

After thawing out the stored pellets, 2 volumes of cold fluorocarbon (Freon 113, Du Pont, Wilmington, Del.) were added and the mixture homogenized for 5 min at 10 000 rpm in a Virtis 60K homogenizer. After phase separation by centrifugation for 10 min at 15 000 rpm (Beckman SW41 rotor), the supernatant was collected and the interphase extracted once more with a small amount of phosphate buffer. The combined aqueous phase was then applied to a 1,5 × 30 cm column of Sephacryl 1000, previously equilibrated at 4 °C with 10 mM phosphate buffer containing 0,5 mM EDTA and 1,0 mM DTT, and eluted in the same buffer.

When required, the semi-purified virus, which eluted immediately after the void volume, was concentrated by pelleting or by vacuum dialysis, using a Millipore CX10 filter unit, and further purified by isopycnic centrifugation in 20–50 % sucrose gradients as described under Results.

RDP assay

All virus concentrations were estimated by means of a standard RDP assay, measuring incorporation of ³H-thymidine triphosphate into DNA by the viral reverse transcriptase, with poly (rA). (dT)₁₀ (Boehringer) as artificial template-primer. Assay buffer (20 mM Tris-HCl, pH 8,3 + 0,33 mM EDTA) was used to dissolve material to be tested. Samples (20 µl) were added to 55 µl of an assay mix to give final concentrations of 14,7 mM Tris, 0,243 mM EDTA, 5 mM MgCl₂ (or 1 mM MnCl₂), 0,24 % (w/v) Triton X-100, 18 mM KCl, 0,3 mM GTP, 14,5 mM DTT, 1,52 µM ³H-TTP (40–60 Ci/mmol) and 5,25 µg of template per assay. After incubation at 37 °C for 20 min, the reaction was terminated by spotting on DEAE cellulose filter discs (Whatman DE81). These were then dried, rinsed 6 times with freshly prepared 5 % Na₂HPO₄ solution and twice with distilled water, dried and counted in a toluene-based scintillation solution in a Beckman L9000 scintillation counter. Virus concentrations were expressed in terms of RDP units, which were equivalent to the total cpm incorporated by the sample.

Protein determination

Protein concentrations were determined by the Peterson modification of the Lowry method (Peterson, 1977).

Polyacrylamide gel electrophoresis

Gel electrophoretic analysis of viral proteins was carried out in 0,1 % SDS-containing 12,5% polyacrylamide gels with a 4 % stacking gel according to the method of King & Laemmli (1971). Viral samples were dissociated by heating in a boiling water-bath for 3 minutes in 2,5 % SDS, 5,0 % β-mercaptoethanol and 12,5 % glycerol.

The low molecular mass calibration kit supplied by Pharmacia Fine Chemicals AB, Uppsala, Sweden, was used as molecular mass markers, run simultaneously.

SDS-Sucrose gradient analysis

Density gradients of 10–30 % sucrose were prepared, using RNasefree sucrose in TNE buffer (10 mM Tris-HCl, pH 7,4, 0,15 M NaCl, 1 mM EDTA) containing 0,5 % SDS. Viral samples were disrupted by adding 0,5 % SDS and heating briefly at 60 °C before layering on the gradient. Centrifugation was for 90 min at 40 000 rpm (SW41 rotor). Drops were collected from the bottom of the tube and absorbance values at 260 and 280 nm determined spectrophotometrically.

Endogenous transcription

Purified JSRV was tested for transcription of its RNA in 500 µl of a reaction mixture containing 50 mM Tris-HCl pH 8,0, 0,024 % Triton X-100, 5 mM DTT, 3,4 mM MgCl₂, 1,8 mM each of dATP, dGTP and dCTP, 50 µg of actinomycin D, 5 µCi ³H-TTP (40–60 Ci/mmol) and 250 µg of activated calf thymus DNA as primer. Incubation at 37 °C was for 3–6 h and the product was analysed on a SDS-sucrose gradient after the addition of 0,5 % SDS.

Source of antisera and conjugates

Antisera against the various retroviruses were prepared in rabbits by injecting virus, purified as described above and disrupted by the addition of 0,25 % Tween X-100. The first injection with Freund's adjuvant was given subcutaneously, followed by boosters at two-weekly intervals without adjuvant in the footpads until a suitable level of neutralizing antibodies was attained. IgG was isolated by chromatography on a DEAE-cellulose column, eluting in an 0,01 M phosphate buffer, pH 6,3. Antiserum to JSRV was prepared in the same way, using fluorocarbon extracted lung rinse pellet, dissociated with Triton X-100, as antigen. Antisera against CAEV and maedi-visna virus were kindly supplied by Dr Travis McGuire, Animal Disease Research Unit, Washington State University, Pullman. Anti-MPMV p27 serum was donated by Dr D. K. Howard, Meloy Laboratories, Inc., Springfield.

Rabbit anti-bovine IgA was purchased from Pel-Freez Biologicals (Rogers, Arkansas) and rabbit anti-sheep IgG from Dakopatts (Copenhagen, Denmark). The anti-bovine IgA gave a specific reaction with sheep IgA. Peroxidase conjugates of anti-rabbit and anti-sheep immunoglobulins, used for the assay of JSRV antigens, IgG and IgA, were also obtained from Dakopatts. For the comparative serological study, conjugates of alkaline phosphatase (type VII, Sigma Chemical Company) and goat anti-rabbit as well as rabbit anti-sheep immunoglobulins were prepared by a modified one step glutaraldehyde method (Avrameas, 1969).

Absorption of antisera

Antiserum prepared against JSRV was extensively absorbed with sheep liver powder, monolayers of foetal lamb kidney cells, Mycoplasma arginini, M. ovipneumonia, sheep IgG and bovine IgA until specific for the virus. The soluble antigens were absorbed to plastic cell culture flasks as described for the ELISA antigens. After being washed thoroughly, the antiserum was added and incubated overnight at 4 °C with constant shaking.

Other antisera were adsorbed, when necessary, with the relevant antigens using the same technique.

RDP neutralization assay

Serial dilutions of the various antiviral IgG preparations were incubated in a total volume of 20 μ l with constant amounts of disrupted purified virus for 30 min at 4 °C, before being assayed for RDP activity as described above.

Normal rabbit IgG controls were tested at comparable concentrations for determining percentage neutralization.

Enzyme linked immunosorbent assay (ELISA)

A modification of the indirect ELISA technique described by Voller, Bidwell & Bartlett (1979) was used both for analysing the relative amounts of viral antigens and immunoglobulins during viral purification, and for a serological comparison of JSRV with other retroviruses. For the former, antigens were bound to microtitre plates (Linbro or Nunc) in twofold dilutions commencing at 1:50 in 0.05 M carbonate buffer at pH 9.6. For the latter, the following dilutions were used in the same buffer: JSRV 1:400; MMTV 1:200; MPMV 1:200; CAEV 1:400; BLV 1:400. After extensive washing with 0.001 M phosphate buffer and 0.05 % Tween 20, the plates were blocked with 3 % ovalbumin in PBS for 1 h at room temperature. This was followed by incubation for 1 h at 37 °C with the primary antiserum. For the quantitation of immunoglobulins and JSRV antigens, anti-sheep IgG was used at a dilution of 1:400, anti-bovine IgA at 1:500 and anti-JSRV at 1:100. For the comparative study, primary antisera were added in twofold dilutions.

After washing, the plates were incubated with the relevant conjugate (as indicated above) for 1 h at 37 °C and washed again before addition of the substrate. The substrate used for the peroxidase conjugate consisted of 0.6 % O-phenylenediamine with 0.3 % hydrogen peroxide in a 0.05 M phosphate-citrate buffer at pH 5.0. The reaction was stopped after about 10 min with 2N H₂SO₄ and the absorbance read at 495 nm in a Titertek Multiscan spectrophotometer. For the alkaline phosphatase conjugates the substrate was 0.1 % p-nitrophenyl phosphate in 0.05 M carbonate buffer at pH 9.6. The reaction was stopped with 2N NaOH and the absorbance read at 405 nm.

Immuno-blot assay

Viral proteins were identified immunologically by electroblotting them after polyacrylamide gel electrophoresis onto nitrocellulose, using a Bio-Rad Trans-Blot apparatus, followed by staining with an enzyme-linked immune reagent. The Bio-Rad Immuno-Blot assay kit was used for this staining reaction in conjunction with an absorbed rabbit anti-JSRV serum at a 1:50 dilution.

RESULTS

Isolation of JSRV from lung-rinse fluids

All attempts to cultivate the jaagsiekte retrovirus in a variety of cell-cultures, applying a wide range of techniques such as co-cultivation, cell fusion and various chemical treatments, have been unsuccessful to date. The only source of virus therefore remained the lungs of adenomatous sheep. Experimental cases of jaagsiekte were produced by injecting new-born lambs intratracheally with semi-purified virus obtained from previous cases. The lambs were slaughtered during the terminal stage of the disease, usually 2–3 months later, and the rinse fluid obtained from the freshly removed lungs used

for the purification procedure described under Materials and Methods. Initial concentration of the lung rinse fluid was usually done by pelleting in a batch rotor. Precipitation with 10 % polyethyleneglycol in 0.5 M NaCl gave similar yields, but it resulted in a heavy precipitation of contaminating proteins which interfered with subsequent steps.

Purification

We have shown previously that treatment of lung rinse pellets with fluorocarbon not only removes much of the contaminating cell debris but also enhances the infectivity of the material (Verwoerd *et al.*, 1980). This was rather surprising as all the other retroviruses tested are very susceptible to an identical treatment, as indicated by a loss of RDP activity. JSRV, in contrast, consistently had a higher RDP activity after treatment than before (Table 1).

TABLE 1 Susceptibility to fluorocarbon treatment

Virus	RDP activity before treatment (cpm)	RDP activity after treatment (cpm)	% inactivation
MuSV (Type C)	41 880	2 100	94.9
SMRV (Type D)	234 630	70 670	69.8
MMTV (Type B)	37 700	2 670	92.9
BLV	59 290	8 030	86.4
JSRV	20 490	28 790	0

This increase in activity was thought to reflect the removal of some inhibitor, possibly immunoglobulin. The phenomenon was further investigated by determining the size distribution of viral RDP activity by gel filtration through a Sephacryl 1000 column before and after treatment with freon. Column fractions were also assayed for relative IgG and IgA concentrations by means of the ELISA technique. The results are shown in Fig. 1.

Untreated lung rinse pellets eluted from the column as a series of RDP positive peaks reflecting a heterogeneous size distribution. IgA and IgG distribution patterns were very similar to that of the virus and little or no activity eluted at the position of free protein (Fig. 1A). After freon treatment, all the RDP activity was found in 2 peaks, respectively eluting immediately after the void volume of the column and in the position of free protein. IgA was present in both peaks, but IgG was found mainly in the free protein position (Fig. 1B).

These results suggest a predominance of virions associated with immunoglobulins (mainly IgA) and possibly other cell components in the untreated lung rinse pellets. Freon treatment disrupts most of the smaller complexes and also some of the virions, but has little effect on the large JSRV-IgA immune complexes which can be isolated in almost pure form by gel filtration as reflected in the polypeptide pattern shown in Fig. 3.

Removal of immunoglobulins

Various attempts were made to dissociate the immune complexes obtained after freon treatment and gel filtration and to remove the IgA and IgG in order to obtain pure JSRV. In view of the instability of the virus a technique was devised which allows very brief treatment of the complexes under controlled conditions.

Reagents commonly used for the dissociation of immune complexes were layered on top of sucrose density gradients and the viral complexes allowed to sediment

through these layers to isopycnic equilibrium. Without any treatment, viral complexes banded at a density of 1,185 g/ml, as measured by the distribution of RDP activity. Sedimentation through an alkaline (pH 11,5) layer or through 6 M guanidine hydrochloride completely disrupted the virus (results not shown). An acid layer (0,02N HCl, pH 2,5) did not affect the density or the RDP activity of the virus whereas 4 M guanidine hydrochloride and 4 M urea resulted in a reduction of RDP activity and in a slightly lower density of 1,17 g/ml.

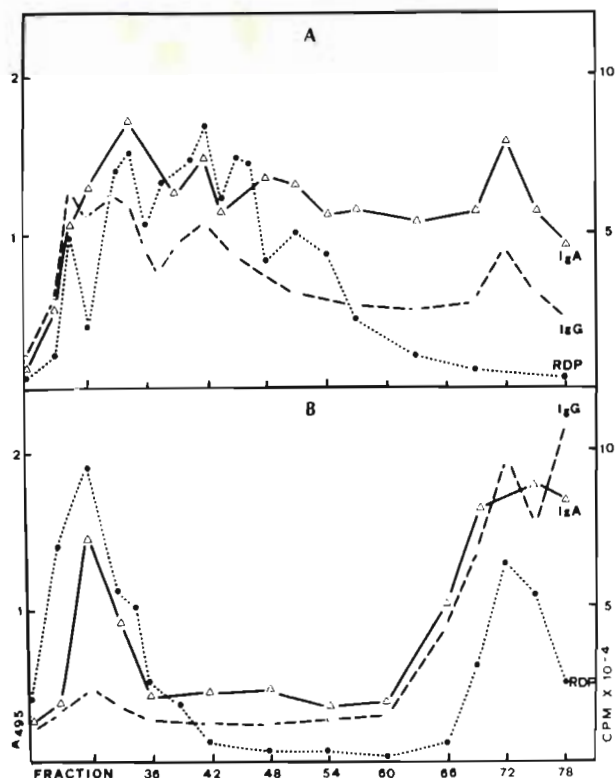


FIG. 1 Effect of freon treatment on the distribution of JSRV-RDP activity, IgA and IgG after gel filtration through a Sephacryl 1000 column. In A a lung rinse pellet was applied to the column without further purification. In B an identical sample was extracted with 2 volumes of freon 113 at 4 °C before application to the column. RDP activity (●) was measured as incorporation of ³H-thymidine triphosphate into DNA with poly (rA). (dT)₁₀ as primer-template. IgG (○) and IgA (Δ) concentrations were determined by means of an ELISA test

The efficiency of the various treatments and purification steps in removing immunoglobulins from the virus was determined by measuring the relative amounts of viral antigen, IgA and IgG by means of the ELISA technique. The results are shown in Fig. 2.

In essence, Fig. 2 confirms that the product of our purification procedure consists of an immune complex of virus and mainly IgA. Isopycnic centrifugation under dissociating conditions reduced the IgG to negligible levels, but we did not succeed in removing all IgA.

A comparison of 2A and 2B shows a considerable reduction in the ratio of IgG and IgA to viral antigen in lung rinse pellets after fluorocarbon extraction. The increase in relative IgA concentration following gel filtration (Fig. 2C) probably reflects the presence of non-viral immune complexes and/or the separation of immune complexes from soluble viral antigens resulting from viral disruption by the fluorocarbon. Fig. 2D indicates that most of the IgG and some of the IgA are removed by isopycnic centrifugation. Acid treatment had little or no additional effect (Fig. 2E). A chaotropic salt layer (4 M

guanidine-HCl or 4 M urea) (Fig. 2F) reduced the relative amount of IgA, but the former also reduced the titre of the viral antigen considerably.

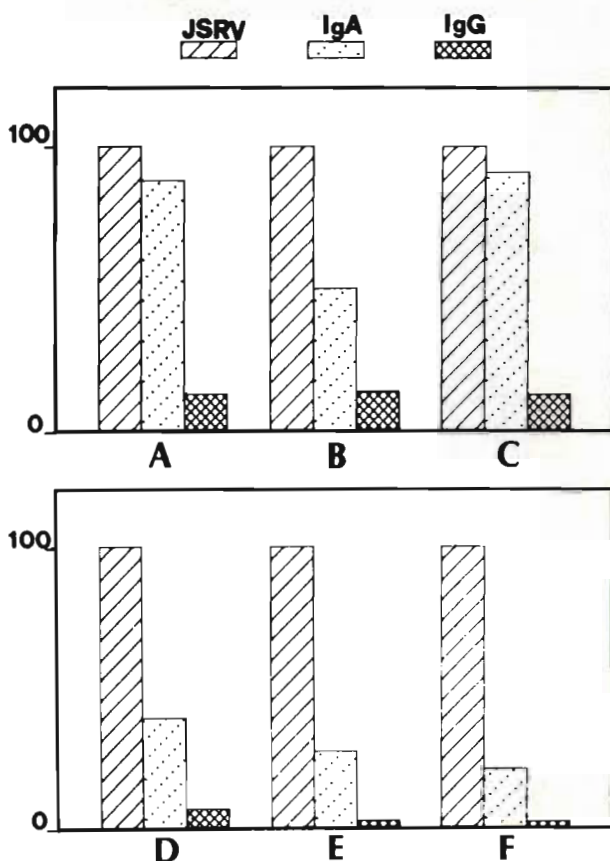


FIG. 2 Relative amounts of JSRV antigen, IgA and IgG at the different stages of purification of the lung rinse pellets: without further purification (A), after freon extraction (B) and fraction 29 from a Sephacryl 1000 column (Fig. 1 B) (C). D represents the viral band obtained by isopycnic density gradient centrifugation, and E and F identical samples sedimented through gradients with additional 1.0 ml layers of 0.02 N HCl in 10 % sucrose (E) and 4M urea (F), respectively. Viral bands were located by means of the RDP test and serial twofold dilutions of the peak fraction used as antigen in the ELISA test. Relative amounts of viral antigen, IgA and IgG were obtained by determining the end-points and expressing the reciprocals of the titres as a percentage of the value for JSRV

Polypeptide composition of the virion

In addition to the assays for immunoglobulins and viral antigens the degree of purification obtained by the procedures described above was also monitored by polyacrylamide gel electrophoretic analysis after each step. The result of a typical experiment is shown in Fig. 3.

The large amount of material removed during the fluorocarbon extraction step is not reflected in Fig. 3 (lanes A and B) and is therefore mainly non-protein in nature. Except for the presence of immunoglobulins, a consistent viral polypeptide pattern was obtained after fluorocarbon treatment followed by gel filtration on Sephacryl 1000, indicating that the RDP-containing peak eluting after the void volume of the column consists mostly of viral immune complexes (lane C). Some minor bands were eliminated by isopycnic centrifugation (lane D) and sedimentation through an acid (lane E) and guanidine HCl layer (lane F). The results obtained with the latter treatments were somewhat variable in various experiments.

Nine polypeptide bands were consistently present in a large number of purified JSRV preparations. The estimated molecular masses are shown in Fig. 3. A tenth

band with a molecular mass of 35 000 daltons was often lost in the final purification steps and may represent a contaminant. A 25 000 band was usually barely visible.

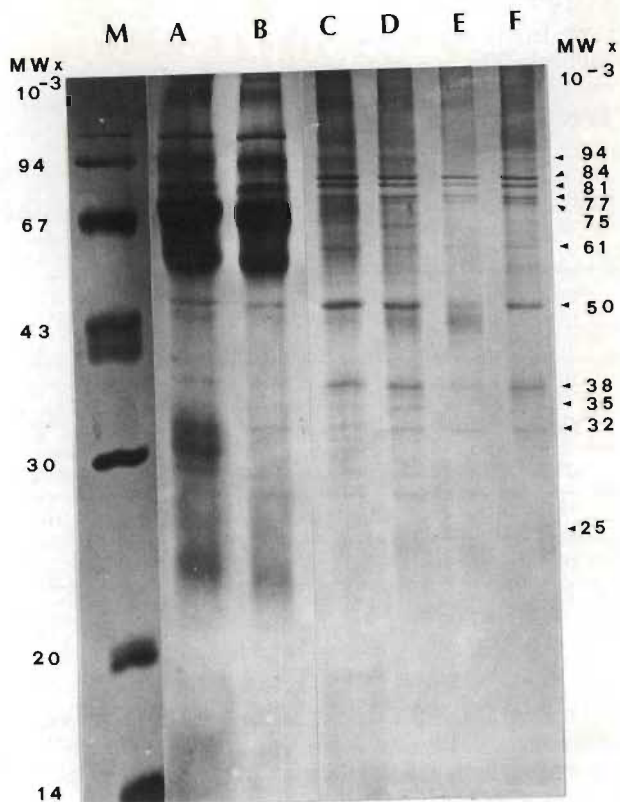


FIG. 3 Polypeptide composition of JSRV at the various stages of purification described in the legend to Fig. 2. Polyacrylamide gel electrophoresis was carried out in a 0.1 % SDS-containing 12.5 % polyacrylamide gel with a 4 % stacking gel run for 12 h at 10.2 V/cm. Lane M represents molecular mass markers (Pharmacia), lanes A-F the fractions illustrated in Fig. 2

In order to obtain some positive identification of the viral polypeptides, a Trans-Blot of the electrophoretic pattern was stained by means of an ELISA-type reaction (Fig. 4A). A cytoplasmic extract from a normal sheep lung was included as a control, and an identical blot was stained with amido black to obtain the complete pattern of polypeptides (Fig. 4B). The bands representing polypeptides p25, p32, p38, p50 and p94 can clearly be seen in the viral antigen after the enzyme-immune reaction (lane J) but were absent from the normal lung extract (lane N). The two double bands in the region of 75–84 000 daltons did not transfer well but were faintly visible on both the stained and immune-reacted blots. These nine bands can therefore be assumed to represent viral polypeptides. In contrast, p35 is clearly a cell component to such an extent that no definite conclusion can be made regarding its identity.

Size of the RNA genome

Previously reported characteristics of the virus, such as the possession of a reverse transcriptase and a density of 1.175, suggested that it would probably have a genome typical for the retroviridae, i.e. a 60–70 S single-stranded RNA. This was confirmed by both direct analysis of the viral genome to determine its size and by the simultaneous assay which demonstrates the transcription of viral RNA to form an RNA-DNA complex of approximately the same size as the RNA template. The results are shown in Fig. 5. Semi-purified virus was dissociated with 1 % SDS and analysed by means of

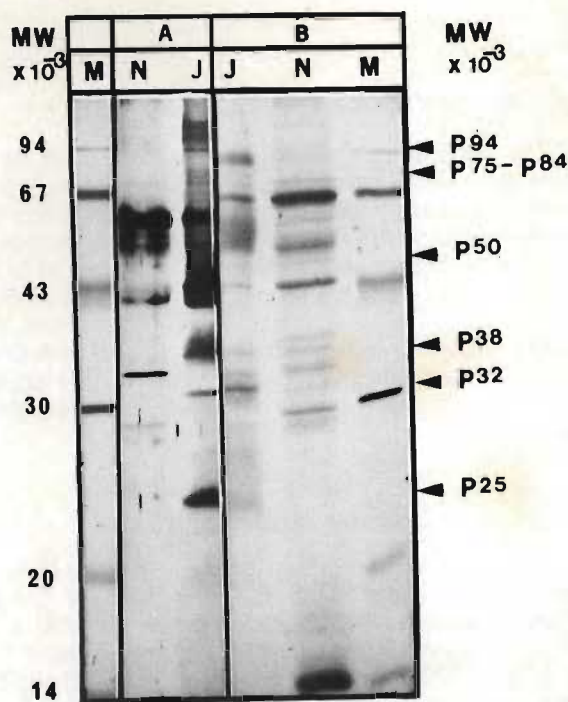


FIG. 4 An immuno-blot assay of the electrophoretically separated polypeptides in a semi-purified JSRV preparation from jaagsiekte lungs (J) and a cell-free extract from normal lungs (N). Lanes M are markers (Pharmacia), with their molecular mass values indicated on the left hand side of the figure. A indicates staining by means of an enzyme-linked immune reagent, B staining with amido black. The polypeptide bands seen only in the viral pattern after the immune reaction are labelled in the right hand lane according to their estimated molecular sizes.

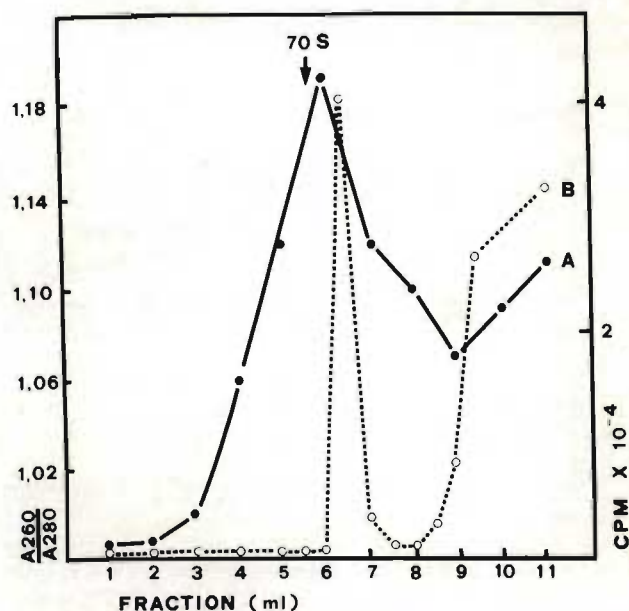


FIG. 5 Evidence for the presence of a 70S RNA genome in JSRV, obtained by means of rate zonal centrifugation in SDS-containing sucrose gradients. A: Purified virus was dissociated with SDS and analysed on an SDS containing gradient as described under Materials and Methods. Fractions (1.0 ml) were collected by means of a peristaltic pump from the bottom of the tube (left side in the figure) and the absorbance measured at 260 and 280 nm (●). The 70S marker indicated by an arrow was a ³H-uridine labelled MuSV dissociated and centrifuged in the same way in a parallel tube. B: An identical JSRV sample was used in an endogenous transcription reaction (described under Materials and Methods), in which ³H-TTP is incorporated into an RNA-DNA hybrid. The sedimentation rate of this product was determined by dissociation and sedimentation as described. Fractions (0.5 ml) were collected from the bottom of the tube and the acid precipitable counts determined in a scintillation counter (○).

sedimentation through an SDS-containing sucrose gradient. Fractions were collected and the absorbance at 260 and 280 nm determined. The nucleic acid component peaked at a position very close to that of a tritium labelled MuSV-RNA used as a 70S marker (Fig. 5A).

The endogenous transcription reaction, carried out as described under Materials and Methods, was very inefficient. This was probably due to inhibitors present in the semi-purified virus, as it was found that the addition of virus to a standardized transcription reaction, utilizing isolated ribosomal RNA as template, reduced transcription to about 1 % of its normal level. Nevertheless, the number of counts incorporated was sufficient to demonstrate that the product of the endogenous reaction yielded a product of approximately the same size as the viral genome (Fig. 5B).

Serological relationship to other retroviruses

In a preliminary attempt to determine whether JSRV is related to any of the established retrovirus types, neutralization of its reverse transcriptase was selected as a group specific test. Antisera against representatives of types B (MMTV), C (MuSV), and D (SMRV) and the unclassified bovine leukaemia virus were prepared and the IgG isolated as described under Materials and Methods. Neutralization curves were derived using each IgG against its homologous enzyme and against the JSRV enzyme. As shown in Fig. 6, no cross-neutralization was obtained.

An indirect ELISA technique was used to confirm and extend these results at a higher level of sensitivity. In addition to representatives of the established types, caprine arthritis encephalitis virus and maedi-visna virus of sheep were also included in the series.

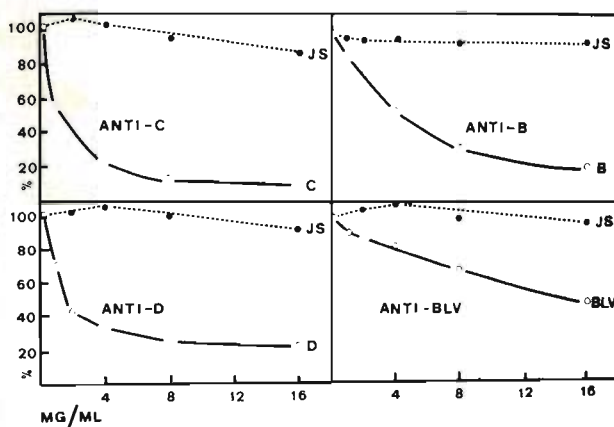


FIG. 6 Neutralization of RNA-dependent DNA polymerases by IgG. Serial dilutions of IgG, prepared in rabbits against type C (MuSV), type B (MMTV), type D (SMRV) and BLV, as well as normal rabbit IgG, were pre-incubated with constant amounts of disrupted purified virus. RDP activity was then determined in triplicate as described under Materials and Methods. Each IgG was tested against its homologous virus and JSRV. Residual RDP activity is expressed as a percentage of that obtained after pre-incubation with an equivalent amount of normal IgG.

The results obtained are presented in Table 2 in the form of a checker-board, where the determinant of the matrix is formed by the titre of each antigen with its homologous serum. The titres for JSRV, MMTV and CAEV along the determinant are 320, while values >640 were obtained for MPMV and BLV. The titrations

of heterologous sera against JSRV-Ag all gave titres below 10 (top row), while titration of JSRV serum against the other viruses also gave titres lower than 10 (left hand column). The weak reactions between the MMTV and SMRV antigens and BLV antiserum were considered to be negative in view of the negative reciprocal titres. The titre of 40 between CAEV-Ag and maedi-visna antiserum is in line with the known cross-reactivity between these 2 viruses.

TABLE 2 Antiserum titres against homologous and heterologous retroviruses as determined with the indirect ELISA technique

Ab \ Ag	JSRV	MMTV	MPMV	CAEV	BLV	MVV
JSRV	320	< 10	< 10	< 10	< 10	< 10
MMTV	< 10	320	< 10	< 10	20	< 10
SMRV	< 10	< 10	> 640	< 10	20	< 10
CAEV	< 10	< 10	< 10	320	< 10	40
BLV	< 10	< 10	10	< 10	> 640	< 10

DISCUSSION

The fact that JSRV had to be isolated from affected adenomatous lungs introduced a number of problems not usually encountered in the purification of retroviruses. In addition to low concentrations of virus and unusual instability after exposure to sucrose or glycerol gradients, the main problem was the presence of large amounts of surfactants, immunoglobulins and cell debris. Our initial demonstration of a viral reverse transcriptase as well as electron microscopic identification of viral particles (Verwoerd *et al.*, 1980) depended on the discovery that much of this contaminating material can be removed by fluorocarbon extraction without apparent damage to the virus. In fact, both reverse transcriptase activity and infectivity of the virus are enhanced by this treatment. This observation was unexpected because typically retroviruses are sensitive to fluorocarbons. Only in the purification of MMTV from blood has freon extraction been used previously (Moore, Sarkar & Charney, 1970). A comparison carried out for a number of representative retroviruses (Table 1) revealed a significant difference between JSRV and the others.

During the course of purification experiments, it became clear that at least some of the virions are present in the lung exudate in the form of immune complexes, mainly with IgA. In fact, only these immune complexes survive the fluorocarbon treatment and constitute the form in which we purified the virus. This explains the resistance of our virus isolates to freon, compared to the other retroviruses, which, of course, were purified from cell cultures in the absence of antibodies. The increase in RDP activity observed after fluorocarbon extraction can be explained in terms of the removal of contaminating substances inhibiting the enzyme reaction. Evidence for such inhibitors was obtained in our study of the endogenous transcription reaction. The presence of inhibitors, and the fact that the RDP assay was the only test for viral activity available to us, made any attempt to estimate yields or even the ratio of complexed to free virus impossible. If the majority of virions present in the lung fluid is complexed with IgA, as suggested by the gel filtration experiments, it would explain the low infectivity of the

virus in nature as well as for cell-cultures and also the fact that secondary spread of lesions in affected lungs is only rarely seen. It would also imply that the immune response of the host is mainly an IgA-mediated local response. This would have important implications for a strategy to combat the disease.

Absolute purity of the virus was not attained by the procedures described. Nevertheless, enough virus of sufficient purity could be obtained in this way for a preliminary study of both its polypeptide composition and its RNA genome. Polyacrylamide gel electrophoretic analysis of the viral proteins yielded a reasonably consistent pattern consisting of 10–11 bands. Nine of these were identified immunologically as viral components. Some of the high molecular mass bands may represent precursor polyproteins. The molecular sizes of the major polypeptides are distinct from those found in other retroviruses. Type C and D retroviruses characteristically possess a 70–80 000 and a 20–36 000 dalton envelope glycoprotein, a major 27–36 000 dalton internal protein and 3–4 low molecular mass polypeptides ranging from 10 to 20 000 daltons. In MMTV, representative type B virus, the distribution pattern is closer to that found in JSRV, i.e. a gp52, gp36, p28, p23, p14 and a p10 (Stephenson, Devar & Reynolds, 1978). In Maedi-Visna virus a large envelop glycoprotein, gp 135, and 3 internal proteins, p30, p16 and p14, as well as some precursor proteins have been demonstrated (Vigne, Filippi, Quérat, Sauze, Vitu, Russo & Delori, 1982).

The possible significance of the apparent absence of polypeptides smaller than 25 000 daltons for the structure and function of the virus remains to be elucidated. In general, retroviruses synthesize polyproteins which are processed by cleavage during viral maturation (Stephenson *et al.*, 1978). The absence of small polypeptides in JSRV could therefore perhaps be ascribed to the lack of a cleavage mechanism. Freon extraction did not alter the morphology of the surviving virus and the small polypeptides are mainly internal components, therefore it is unlikely that they were removed by this treatment.

In contrast to its polypeptide composition, the RNA genome of JSRV does not seem to have any unusual features. It is 60–70S in size and is efficiently transcribed *in vitro* into DNA using exogenous AMV-reverse transcriptase (Verwoerd, unpublished results, 1983). In contrast, the endogenous transcription was very inefficient in our hands, but it was shown experimentally to be due to a contaminating inhibitor.

The magnesium dependence of its reverse transcriptase, its polypeptide composition as well as its morphology and morphogenesis (Payne, Verwoerd & Garnett, 1983), suggested that JSRV could be more closely related to MMTV than to the other retroviruses. To investigate any possible relationships, 2 serological techniques were used. Neutralization of the viral reverse transcriptase has been widely used to study the relationship between various avian and mammalian retroviruses (Bauer & Temin, 1979; Livingston & Todaro, 1973; Smith, Nooter, Bentvelzen, Robert-Guroff, Horewood, Reitz, Lee & Gallo, 1979). We were unable to produce an antiserum active against JSRV-RDP, but antibodies neutralizing the enzymes of representatives of the various groups of retroviruses failed to inhibit the JSRV enzyme. In a more sensitive enzyme immunoassay the same viruses were tested reciprocally against each other. Again no relationship was found. Two additional viruses were included in this series, i.e. maedi-visna and caprine arthritis-encephalitis viruses, representing

the lentivirus subgroup of retroviruses occurring in sheep and goats. Except for a known cross-reaction between the latter 2 viruses, the results were again negative.

As far as we know maedi-visna does not occur in South Africa. We were therefore unable to carry out a direct comparison between maedi-visna virus and JSRV. However, a large number of sheep sera, including those from most of our jaagsiekte cases, were kindly tested for maedi-visna antibodies by Dr Peturrson of Reykjavik, Iceland. No maedi-visna antibodies were found. Conversely, as reported in this paper, antisera against both maedi-visna and CAEV did not react with JSRV antigen. Most importantly, however, the morphology and morphogenesis of the two viruses are markedly different, as discussed in the accompanying paper (Payne *et al.* 1983). We therefore conclude that JSRV is probably a new retrovirus, unrelated to any of the previously known members of this group.

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