Onderstepoort J. vet. Res., 50, 309–316 (1983)

ISOLATION AND PRELIMINARY CHARACTERIZATION OF THE JAAGSIEKTE RETROVIRUS (JSRV)

D. W. VERWOERD, ANNA-LISE PAYNE, D. F. YORK and M. S. MYER, Veterinary Research Institute, Onderstepoort 0110

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 domonstrated 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.

Received 9 September 1983—Editor

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, Bat_2-$C1_1$, 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-HC1, pH 7,5, 5 mM $MgC1_2$, 0,5 mM EDTA, 0,1 M KC1 and 1 mM DTT), and stored at −70 °C until used. Preliminary purification was obatined 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 isopyknic 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 isopyknic 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 ^{3}H-thymidine triphosphate into DNA by the viral reverse transcriptase, with poly (rA). $(dT)_{10}$(Boehringer) as artificial template-primer. Assay buffer (20 mM Tris-HC1, 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 $MgC1_2$ (or 1 mM $MnC1_2$), 0,24 % (w/v) Triton X-100, 18 mM KC1, 0,3 mM GTP, 14,5 mM DTT, 1,52 μM ^{3}H-TTP (40–60 Ci/mmole) 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 % Na_2HPO_4 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-HC1, pH 7,4, 0,15 M NaC1, 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-HC1 pH 8,0 0,024 % Triton X-100, 5 mM DTT, 3,4 mM $MgC1_2$, 1,8 mM each of dATP, dGTP and dCTP, 50 μg of actinomycin D, 5 μCi ^{3}H-TTP(40–60 Ci/mmole) 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-Freeze 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).

Absorbtion 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 $\mu\ell$ 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 H_2SO_4 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 indentified 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 Immun-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 isopyknic 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 HC1, 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.

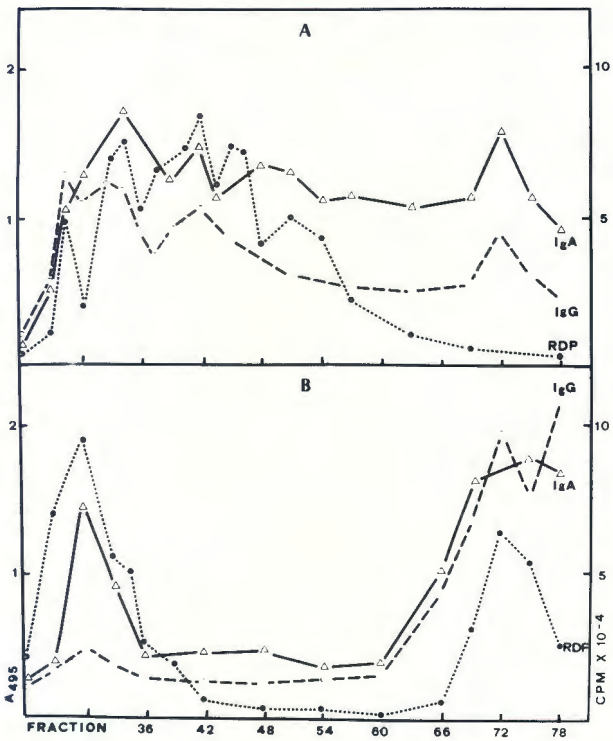

FIG. 1 Effect of freon treatment on the distribution of JSRV-RDP activity, IgA and IgG after gel filtration through a Sephacryl 1000 column. In A a lung rinse pellet was applied to the column without further purification. In B an identical sample was extracted with 2 volumes of freon 113 at 4 °C before application to the column. RDP activity (●) was measured as incorporation of ^{3}H-thymidine triphosphate into DNA with poly (rA). $(dT)_{10}$ as primer-template. IgG (o) 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. Isopyknic 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 isopyknic centrifugation. Acid treatment had little or no additional effect (Fig. 2E). A chaotropic salt layer (4 M guanidine-HC1 or 4 M urea) (Fig. 2F) reduced the relative amount of IgA, but the former also reduced the titre of the viral antigen considerably.

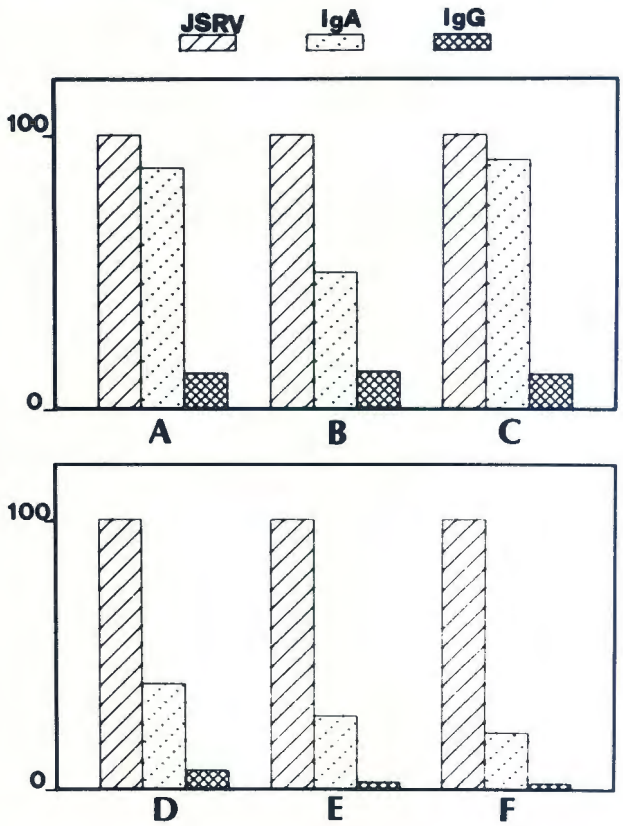

FIG. 2 Relative amounts of JSRV antigen, IgA and IgG at the different stages of purification of the lung rinse pellets: without further purification (A), after freon extraction (B) and fraction 29 from a Sephacryl 1000 column (Fig. 1 B) (C). D represents the viral band obtained by isopyknic density gradient centrifugation, and E and F identical samples sedimented through gradients with additional 1,0 mℓ layers of 0,02 N HC1 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 isopyknic centrifugation (lane D) and sedimentation through an acid (lane E) and guanidine HC1 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.

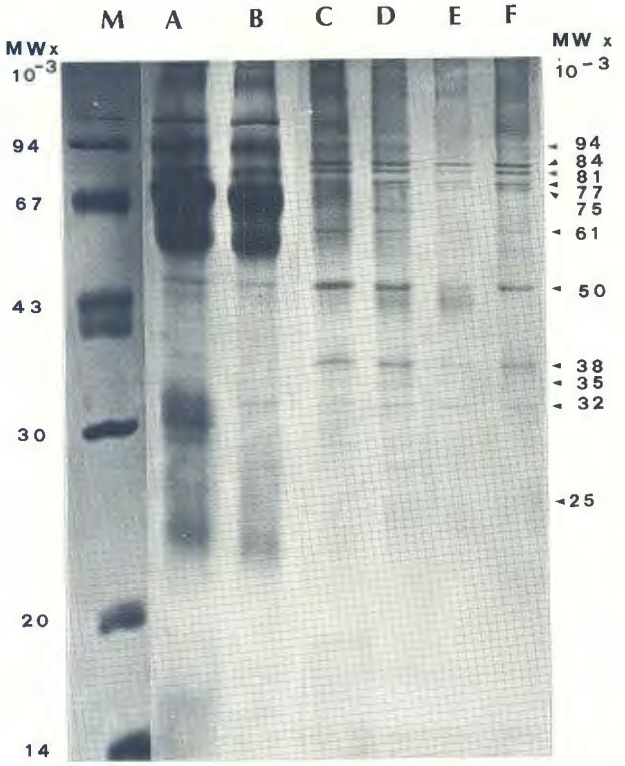

FIG. 3 Polypeptide composition of JSRV at the various stages of purification described in the legend to Fig. 2. Polyacrylamide gel electrophoresis was carried out in a 0,1 % SDS-containing 12,5 % polyacrylamide gel with a 4 % stacking gel run for 12 h at 10,2 V/cm. Lane M represents molecular mass markers (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 and p61 is overshadowed by a normal 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

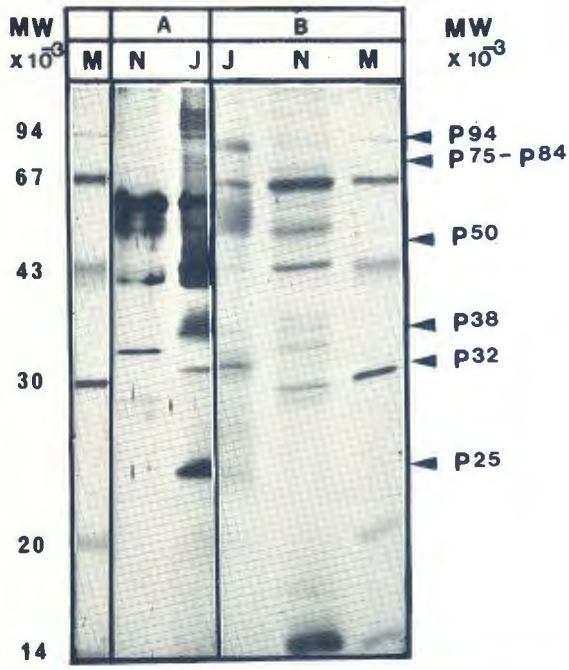

FIG. 4 An immuno-blot assay of the electrophoretically separated polypeptides in a semi-purified JSRV preparation from jaagsiekte lungs (J) and a cell-free extract from normal lungs (N). Lanes M are markers (Pharmacia), with their molecular mass values indicated on the left hand side of the figure. A 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.

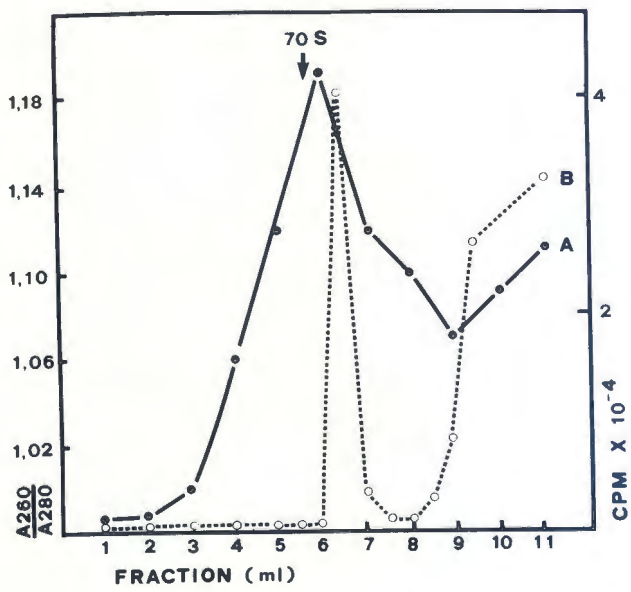

FIG. 5 Evidence for the presence of a 70S RNA genome in JSRV, obtained by means of rate zonal centrifugation in SDS-containing sucrose gradients. A: Purified virus was dissociated with SDS and analysed on an SDS containing gradient as described under Materials and Methods. Fractions (1,0 mℓ) were collected 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 ^{3}H-uridine labelled MuSV dissociated and centrifuged in the same way in a parallel tube. B: An identical JSRV sample was used in an endogeneous transcription reaction (described under Materials and Methods), in which ^{3}H-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.

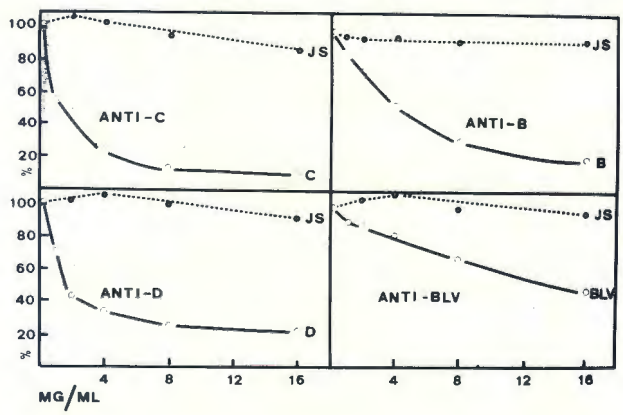

FIG. 6 Neutralization of RNA-dependent DNA polymerases by IgG. Serial dilutions of IgG, prepared in rabbits against type C (MuSV), type B (MMTV), type D (SMRV) and BLV, as well as normal rabbit IgG, were pre-incubated with constant amounts of disrupted purified virus. RDP activity was then determined 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

Ag \ Ab	JSRV	MMTV	MPMV	CAEV	BLV	MVV
JRSV	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, Filipi, 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 antobodies 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.

Acknowledgements

We should like to thank Dr G. Peturrson, Institute for Experimental Pathology, Reykjavik, Iceland, for testing a number of sera, Dr J. Ferrer of the University of Pennsylvania, USA, for providing the Bat_2C1_1cell line Dr T. McGuire of the Washington State University, Pullman, USA, for providing us with CAEV antigen and antiserum and Maedi-Visna antiserum, and Dr D. K. Howard Meloy Laboratories Inc., Springfield, USA, for MPMV antiserum.

References

AVRAMEAS, S., 1969. Coupling of enzymes to proteins with glutaraldehyde. *Immunocytochemistry*, 6, 43–52.

BAUER, G. & TEMIN, H. M., 1979. RNA-directed DNA polymerase from particles released by normal goose cells. *Journal of Virology*, 29, 1006–1013.

DE VILLIERS, ETHEL-MICHELE & VERWOERD, D. W., 1980. Presence of *Herpesvirus ovis* DNA sequences in cellular DNA from sheep lungs affected with jaagsiekte (pulmonary adenomatosis). *Onderstepoort Journal of Veterinary Research*, 47, 109–112.

HERRING, A. J. SHARP, J. M., SCOTT, F. M. M. & ANGUS, K. W., 1983. Further evidence for a retrovirus as the aetiological agent of sheep pulmonary adenomatosis (jaagsiekte). *Veterinary Microbiology*, 8, 237–249.

KING, J. & LAEMMLI, U. K., 1971. Polypeptides of the tail fibres of bacteriophage T4. *Journal of Molecular Biology*, 62, 465–473.

LIVINGSTON, D. M. & TODARO, G. J., 1973. Endogenous type C virus from a cat cell clone with properties distinct from previously described feline type C virus. *Virology*, 53, 142–151.

MALMQUIST, W. A., KRAUSS, H. H., MOULTON, J. E. & WANDERA, J. G., 1972. Morphologic study of virus-infected lung cell cultures from sheep pulmonary adenomatosis (jaagsiekte). *Laboratory Investigation*, 26, 528–533.

MOORE, D. H. SARKAR, N. H. & CHARNEY, J., 1970. Bioactivity and virions in the blood of mice with mammary tumor virus. *Journal of the National Cancer Institute*, 44, 965–973.

PAYNE, A., VERWOERD, D. W. & GARNETT, H. M., 1983. The morphology and morphogenesis of jaagsiekte retrovirus (JSRV). *Onderstepoort Journal of Veterinary Research*, 50, 317–322.

PERK, K. HOD, I. & NOBEL, T. A., 1971. Pulmonary adenomatosis of sheep (jaagsiekte). I. Ultrastructure of the tumor. *Journal of the National Cancer Institute*, 46, 525–537.

PERK, K., MICHALIDES, R. SPIEGELMAN, S. & SCHLOM, J., 1974. Biochemical and morphologic evidence for the presence of an RNA tumor virus in pulmonary carcinoma of sheep (jaagsiekte). *Journal of the National Cancer Institute*, 53, 131–135.

PETERSON, G. L., 1977. A simplification of the protein assay method of Lowry *et. al.* which is more generally applicable. *Analytical Biochemistry*, 83, 346–356.

SMITH, R. G., NOOTER, K., BENTVELZEN, P., ROBERT-GUROFF, M., HOREWOOD, K., REITZ, M. S. LEE, S. A. & GALLO, R. C., 1979. Characterization of a type-C virus produced by co-cultures of human leukemic bone marrow and fetal canine thymus cells. *International Journal of Cancer*, 24, 210–217.

STEPHENSON, J. R., DEVARE, S. G. & REYNOLDS, F. H., 1978. Translation products of type C RNA tumor viruses. *Advances in Cancer Research*, 27, 1–53.

TUSTIN, R. C., 1969. Ovine Jaagsiekte. *Journal of the South African Veterinary Medical Association*, 40, 3–23.

VERWOERD, D. W. WILLIAMSON, A. & DE VILLIERS, E-M., 1980. Aetiology of Jaagsiekte: transmission by means of subcellular fractions and evidence for the involvement of a retrovirus. *Onderstepoort Journal of Veterinary Research*, 47, 275–280.

VIGNE, R., FILIPI, P., QUÉRAT, G., SAUZE, N., VITU, C., RUSSO, P. & DELORI, P., 1982. Precursor polypeptides to structural proteins of visna virus. *Journal of Virology*, 42, 1046–1056.

VOLLER, A., BIDWELL, D. E. & BARTLETT, A., 1979. The enzyme linked immunosorbent assay (ELISA). A guide with abstracts of microplate applications. Dynatech Laboratories Inc. Alexandria, V. A.

WANDERA, J. G., 1971. Sheep pulmonary adenomatosis (Jaagsiekte). *Advances in Veterinary Science and Comparative Medicine*, 15, 251–283.

Printed by and obtainable from the Goverment Printer, Private Bag X85, Pretoria, 0001