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AETIOLOGY OF JAAGSIEKTE: TRANSMISSION BY MEANS OF SUBCELLULAR FRACTIONS AND EVIDENCE FOR THE INVOLVEMENT OF A RETROVIRUS

D. W. VERWOERD, ANNA-LISE WILLIAMSON and ETHEL-MICHELE DE VILLIERS, Veterinary Research Institute, Onderstepoort 0110

Abstract

VERWOERD, D. W., WILLIAMSON, ANNA-LISE & DE VILLIERS, ETHEL-MICHELE, 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, (1980).

Jaagsiekte (ovine pulmonary adenomatosis) was transmitted to new-born lambs by inoculation of the microsomal fraction of a cytoplasmic extract of cultured tumour cells or tumour tissue. Various treatments of the biologically active fraction were carried out to differentiate between various classes of possible aetiological agents. The results obtained suggested the involvement of a membrane-associated RNA containing virus. Reverse transcriptase activity dependent on Mg^{++} was subsequently demonstrated in these extracts and in lung exudate, and was shown to be associated with particles banding at a density of 1,175 in sucrose gradients. These characteristics, as well as the appearance of the particles in the electron microscope, are similar to those reported for Type B and Type D retroviruses. Serial transmissions of jaagsiekte over a number of years, using cytoplasmic extracts and purified virus, strongly suggest that this virus is the aetiologic agent of jaagsiekte.

Résumé

L'ÉTIOLOGIE DE L'ADÉNOMATOSE PULMONAIRE OVINE (JAAGSIEKTE): TRANSMISSION AU MOYEN DE FRACTIONS SUB-CELLULAIRES ET ÉVIDENCE DE L'IMPLICATION D'UN RETROVIRUS

L'adénomatose pulmonaire ovine à été transmise à des agneaux nouveau-nés par l'inoculation de la fraction microsomale d'un extrait cytoplasmique de cellules de tissus tumoral ou de tissus de tumeur en culture. Des traitements variés de fraction biologiquement active furent réalisés pour différencier entre les classes variées d'agents éventuellement étiologiques. Les résultats obtenus ont suggéré l'implication d'un virus ARN associé à une membrane. Une activité transcriptase inverse dépendante de Mg^{++} a été démontrée par la suite dans ces extraits et dans l'excrétion du poumon et elle se révéla être associée avec des particules en rubans à une densité de 1,175 dans les indicateurs de pentes à sucrose. Ces caractéristiques, aussi bien que l'apparence des particules au microscope électronique sont similaires à celles enrégistrées pour les retrovirus de type B et de type D. Des transmissions en séries d'adénomatose pulmonaire ovine pratiquéés au cours d'un certain nombre d'années, en utilisant les extraits cytoplasmiques et du virus purifié, suggèrent très fortement que ce virus est un agent aetiologique de l'adénomatose pulmonaire.

Introduction

After establishing several tumour cell lines from jaagsiekte (ovine pulmonary adenomatosis) lung tissue, we first reported transmission of the disease by transplantation of intact cells (Coetzee, Els & Verwoerd, 1976). Subsequently, we also demonstrated *in vivo* transformation of the recipients' cells by inoculation of cell-free extracts of these cells (Verwoerd, De Villiers & Tustin, 1980). This confirmed previous reports that the disease can be transmitted experimentally by cohabitation, droplet infection and parenteral inoculations of extracts of diseased lungs, suggesting a viral aetiology (for reviews see Tustin, 1969, and Wandera, 1971).

Viruses have been isolated and incriminated as possible aetiological agents by various workers. Herpes-like viruses were demonstrated in or isolated from adenomatous lungs by Mackay (1969), Malmquist, Krauss, Moulton & Wandera (1972), Cvjetanovic, Forsek, Nevjestic & Rukanvia (1972) and De Villiers, Els & Verwoerd (1975). However, neither transmission nor serological or molecular hybridization experiments yielded any evidence for the involvement of this virus (De Villiers & Verwoerd, 1980).

Biochemical and morphological evidence for the presence of a retrovirus in adenomatous lungs was presented by Perk, Michalides, Spiegelman & Schlom, (1974), while Malmquist *et al.* (1972) demonstrated morphologically typical retroviruses in cell cultures made in the USA from Kenyan material. However, the possibility that these viruses may be identical with the retrovirus causing progressive pneumonia or maedi, a disease known to occur in East Africa, has not been excluded, nor have any transmission studies with these viruses been reported.

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A thorough electron microscopic examination of our cultured tumour cells yielded no evidence of virus particles, nor could we either demonstrate reverse transcriptase (RNA-dependent DNA polymerase or RDP), an enzyme characteristic of retroviruses, in cell homogenates or rescue any defective virus from the cultures. We therefore embarked on a programme of systematic fractionation of tumour cells as well as tumour tissue in our search for the aetiological agent. Fractions were tested for oncogenicity in new-born lambs, and fractions found to be infective were subjected to various treatments in order to differentiate between various classes of possible agents. The better purification procedures developed in the course of these investigations enabled us to isolate and study the organism involved. The results of these studies are reported in this paper.

Materials and Methods

Cell cultures

Cultures of the 15.4 tumour cell line, which was established from jaagsiekte tumour tissue (Coetzee *et al.,* 1976), were cultivated as previously described (Verwoerd *et al.,* 1980). Cells were harvested by trypsinization and disrupted for the isolation of cytoplasmic fractions by swelling for 1 h at 4 °C in hypotonic buffer A (50 mM Tris-HCl pH 7,5; 5 mM $MgCl_2$; 0,5 mM EDTA; 20 mM DTT and 0,1 M sucrose) and freezing and thawing twice before disrupting in a tight-fitting Dounce homogenizer (20 strokes).

Preparation of subcellular fractions from lung tissue

Adenomatous lung tissue from jaagsiekte cases were processed fresh when available or, more often, after storage for up to a year at −70 °C. After thawing, 20 g of tissue was homogenized in 50 mℓ of

buffer A in a Buehler homogenizer. To this 100 mℓ of buffer A was added and the cells were disrupted in a loose-fitting Dounce homogenizer. Various subcellular fractions were isolated by means of differential centrifugation as follows:

Nuclei and large debris: 15 min at 500 × g
Mitochondria and fast sedimenting membranes: 10 min at 13 000 × g
Microsomal fraction: 90 min at 75 000 × g
Post-ribosomal fraction: 16 h at 113 000 × g

Where only the microsomal fraction was required, the first 2 steps were combined and the microsomal fraction pelleted through a 5% sucrose cushion. All pellets were dissolved in isotonic buffer B (50 mM Tris-HCl pH 7,5; 5 mM $MgCl_2$; 0,5 mM EDTA; 100 mM KCl and 1 mM DTT) and stored at −70 °C, if necessary.

Density gradient centrifugation

Isopycnic centrifugation was done in 20–55% sucrose gradients in buffer B, centrifuging in a SW27 rotor for 16 h at 25 000 rpm. Fractions of 1,0 mℓ were collected from the bottom and their densities determined refractometrically. For zonal centrifugation 5–20% sucrose gradients were used in a SW41 rotor spun for 30 min at 30 000. Virus was localized in gradients by testing individual fractions for RDP activity.

Isolation of RNA and DNA

RNA was isolated from the microsomal fraction by either the phenol extraction method commonly used for extracting infective viroid RNA (Raymer & Diener, 1969), or by chloroform isoamyl-alcohol extraction, to eliminate the possibility of losing an RNA covalently attached to a polypeptide in the interphase. Microsomal pellets were dissolved in buffer B containing 0,05% polyvinylsulphate as nuclease inhibitor. It was then extracted 3 times with an equal volume of either water-saturated phenol or a mixture of 24:1 chloroform:isoamylalcohol. Phenol was removed by 4 extractions with freshly distilled ether and the RNA precipitated from the aqueous phases with 2,5 volumes of cold ethanol. RNA and DNA were isolated simultaneously from 15.4 cells by the procedure developed by Wolf (1975). Cells (2×10^8) were suspended in 20 mℓ of 0,01 M Tris-HCl, pH 7,4 to which were added 2,5 mℓ of 10% sarkosyl and 500 μg/mℓ proteinase K. After incubation for 30 min at 37 °C the mixture was extracted twice with an equal volume of saturated phenol, once with 24:1 chloroform:isoamylalcohol and 3 times with ether. After the ether was removed by bubbling with nitrogen gas, 9,0 mℓ of extract was mixed with 1,0 mℓ of a solution containing 0,15 M sodium citrate and 0,1 M sodium metabisulphite, and with 10 g of KI to give a refractive index of 1,4290. The mixture was then centrifuged in a 50 rotor for 72 h at 40 000 rpm. DNA was precipitated from the top fractions and RNA from the bottom fractions with 2,5 volumes of cold ethanol.

Treatment with fluorocarbon

Pellets obtained from cytoplasmic extracts or from rinsing lungs were suspended in buffer B and shaken well with an equal volume of Freon 113 (Dupont) for 15 min at 4 °C. After centrifugation for 10 min at 7 500 rpm the aqueous phase was collected and the interphase extracted with half a volume of buffer B in the same way.

Inoculation of lambs

Dorper lambs less than a week old were used for all oncogenicity tests. Samples to be tested were injected intratracheally in 4–5 mℓ volumes. The lambs were kept under observation as experimental groups in separate closed pens for up to 1 year. When they showed advanced symptoms, or after a year, they were slaughtered and their lungs examined macroscopically and histologically for jaagsiekte lesions.

RDP assay

Assay buffer (20 mM Tris-HCl, pH 8,3+0,33 mM EDTA) was used to dissolve pelleted material to be tested for RNA-dependent DNA polymerase activity. 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 $MgCl_2$ (or 1 mM $MnCl_2$), 0,24% (w/v) Triton X-100, 18 mM KCl, 0,3 mM GTP, 14,5 mM DTT, 1,52 μM ^{3}H-TTP (40–60 Ci/mMole) and 5,25 μg of template(1)/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.

Electron microscopy

Thin sections: Lung rinse pellets were fixed overnight in 3% glutaraldehyde and post-fixed for 1 h in 1% OsO_4, both fixatives being dissolved in 0,1 M sodium cacodylate buffer, pH 7,2 containing 4% sucrose. After dehydration in a graded acetone series the sample was cleared in propylene oxide and embedded in Epon (Luft, 1961). Thin sections were picked up on copper grids, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a Siemens Elmiskop 102 electron microscope. Measurement of particles was done on a Nikon profile projector, model 6C.

Negative staining: A formvar-carbon-coated grid was floated consecutively on drops of virus suspension, 1% OsO_4, distilled water and 3% phosphotungstic acid, pH 6. The time on each droplet varied between 10 seconds and 1 minute.

Results

Transmission of jaagsiekte with various subcellular fractions

Cells derived from jaagsiekte lung tissue were disrupted in a Dounce homogenizer in hypotonic buffer as described under Materials and Methods. The cell homogenates were then fractionated by differential centrifugation into nuclear, mitochondrial, microsomal and post-ribosomal fractions according to Malone, Marsh, Hanson & Semancik (1978), and each fraction was injected intratracheally into new-born lambs as described.

The results summarized in Table 1 clearly indicate that infectivity resides in the microsomal fraction of the cytoplasm containing a large amount of slow-sedimenting membranes. Cells of the 15.4 cell line were fractionated in the same way and again the microsomal pellet proved to be oncogenic (results not shown).

(1) Templates were obtained from Boehringer (SA) Ltd, Johannesburg

TABLE 1 Transmission of jaagsiekte to new-born lambs with subcellular fractions derived from tumour tissue

Centrifugal fraction (pellet)	Contents	No. of positives/ No. inoculated
15 min 500 × g....	Nuclei + cell debris.....	0/4
10 min 13 000 × g..	Mitochondria + membranes	0/2
90 min 75 000 × g...	Membranes + ribosomes...	4/8
16 h 113 000 × g....	Post-ribosomal fraction....	0/2

The biologically active microsomal pellet was then further fractionated according to density by isopycnic centrifugation in a sucrose gradient. Density fractions were collected as indicated in Table 2 and tested for oncogenicity as described above. Activity did not band at a specific density but was distributed throughout the density range in which cellular membranes are found (Table 2). Such an association with cell membranes is commonly found in retroviruses, but it is also characteristic of the scrapie agent, which is thought to consist of a free nucleic acid molecule associated with membranes (Malone, Marsh, Hansen & Semancik, 1979). RNA and DNA were therefore isolated by various procedures (see Materials and Methods) from the 1,16–1,18 lung fraction (Table 2) and from 15.4 cells. No oncogenic activity was found in any of the RNA or DNA isolates tested (Table 2).

TABLE 2 Density distribution of oncogenic activity in the cytoplasm of tumour cells and absence of activity in the nucleic acids isolated from it

Inoculum	No. of positives/ No. inoculated
Cytoplasmic fraction from 15,4 cells or tumour tissue with a buoyant density in sucrose of	
1,10–1,14.....	0/3
1,14–1,16.....	2/3
1,16–1,18.....	2/3
1,18–1,20.....	1/3
Nucleic acid isolated from 1,16–1,18 lung fraction by extraction with	
(a) phenol.....	0/5
(b) chloroform/isoamyl alcohol.....	0/2
RNA isolated from 15,4 tumour cells (KI gradient)	0/5
DNA isolated from 15,4 tumour cells (KI gradient)	0/5

Effect of various treatments on the oncogenic activity of cytoplasmix extracts

A cytoplasmic extract was prepared from a lung tumour (S6338) which had been stored at −70 °C for 6 months and fractionated by isopycnic centrifugation on a sucrose gradient, as described under Materials and Methods. Fractions with densities between 1,14 and 1,18 were pooled and subjected to the various treatments shown in Table 3 before being tested for oncogenicity in new-born lambs, as described above.

The results shown in Table 3 demonstrate that the crude jaagsiekte agent is heat-labile, is completely inactivated by sarkosyl and by aqueous phenol, partially inactivated by non-ionic detergent (NP-40), RNase and proteinase K and not affected by treatment with fluorocarbon or DNase.

TABLE 3 Effect of various treatments on the oncogenic activity of a cytoplasmic fraction from tumour tissue

Treatment	No. of positives/ No. inoculated
No treatment (controls).....	3/4
Heat: 60 min 80 °C.....	0/2
DNase: 20 mg/mℓ, 30 min 37 °C.....	4/4
RNase: 20 mg/mℓ, 30 min 37 °C.....	2/4
Proteinase K: 50 mg/mℓ 30 min 37 °C.....	1/2
Sarkosyl 0,5% + proteinase K 30 min 37 °C.....	0/2
Freon: shaken 15 min 4 °C.....	4/4
Phenol: shaken 2 × 10 min 4 °C.....	0/4
NP-40 1%, 30 min 37 °C.....	1/4

Demonstration of RNA-dependent DNA polymerase (RDP) activity in cytoplasmic extracts and lung exudate

Initial attempts to confirm the presence of the characteristic RDP activity of retroviruses in tumour extracts, as demonstrated by Perk *et al.* (1974), were unsuccessful. Owing to the presence of large amounts of membrane vesicles in the biologically active density fractions it was also impossible to recognize any viral particles in the electron microscope. The discovery that fluorocarbon treatment does not affect the infectivity of cytoplasmic pellets enabled us to purify and concentrate the tumour extracts considerably. A low RDP activity was found in such concentrated extracts, as shown in Table 4 for Sheep 9144.

A characteristic feature of advanced jaagsiekte is the copious secretion of fluid in the affected lungs. Sharp & Herring (personal communication, 1978) reported reverse transcriptase activity in this fluid. Using their assay system (see Materials and Methods), we also found considerably higher enzyme activity in lung fluid than that in lung extracts (Table 4). This result is consistent with the fact that retroviruses are found mainly extra-cellularly, assembly of the virions taking place at the cell surface.

TABLE 4 RNA-dependent DNA polymerase (RDP) activity in fractions isolated from jaagsiekte lungs (Sheep 9144)

Treatment	RDP-activity (dpm/20 μℓ)
Cytoplasmic extract from 20 g of tumour tissue, concentrated 100-fold and fractionated on a sucrose density gradient.....	2 400
Lung exudate, concentrated about 100-fold, unpurified.....	13 000
Same, filtered through 450 nm Millipore filter....	17 600
Same, filtered through 200 nm Sartorius filter.....	16 100
Same, filtered through a 100 nm Sartorius filter....	310
Same, witn Mn^{++} replacing Mg^{++} in standard mix	498
Same, without template.....	380
Same, with poly(dA)-oligo(dT) replacing poly (rA)-oligo (dT) as template.....	195
Same, with poly (rC)-oligo (dG) as template.....	4 200

In order to prove its identity as a viral polymerase, some characteristics of the RDP activity found in lung fluid were determined. The results shown in Table 4 illustrate that the enzyme is associated with a particle having a diameter of between 80 and 150 nm, prefers Mg^{++} above Mn^{++} for the templates tested and can utilize poly (rA)-oligo (dT) and poly (rC)-oligo (dG) but not poly (dA)-oligo (dT) as

template. Fig. 1 shows that the RDP activity bands at a buoyant density of 1,175 in sucrose gradients. These characteristics strongly suggest the presence of a retrovirus in the lung secretion.

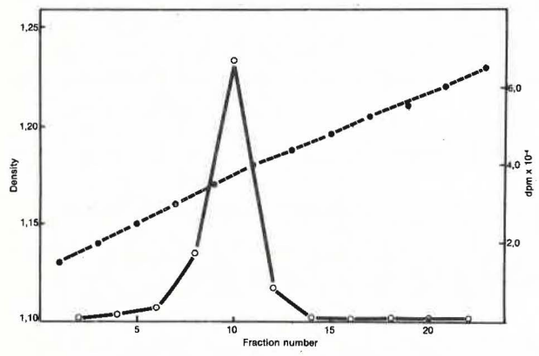

FIG. 1 Buoyant density of the RDP-active particles obtained from the lung rinse of sheep 9144. A dissolved pellet was layered without further treatment on a 20–55% sucrose gradient and centrifuged for 16 h at 24 000 rpm in a SW27 rotor

Density • - - - •, RDP activity (dpm/20 $\mu\ell$): o ——— o

Electron microscopic demonstration of viral particles in the lung fluid

Electron microscopy of the peak fractions in Fig. 1 (density 1,17–1,18) revealed electron-dense particles with an average size of about 89 nm and a close-fitting membrane (Fig. 2).

Pleomorphic virus-like particles were observed in negatively stained preparations. The particle membrane was covered with knobbed projections (Fig. 3).

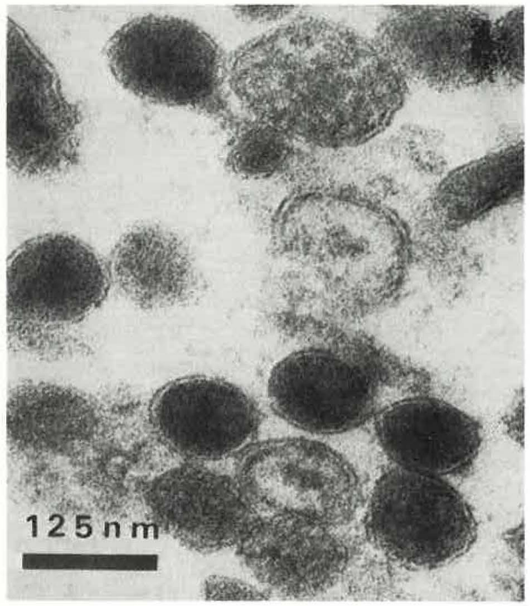

FIG. 2 Electron micrograph of the peak fractions from the gradient shown in Fig. 1 (density 1,17–1,18). After centrifugation the pellets were fixed, sectioned and positively stained

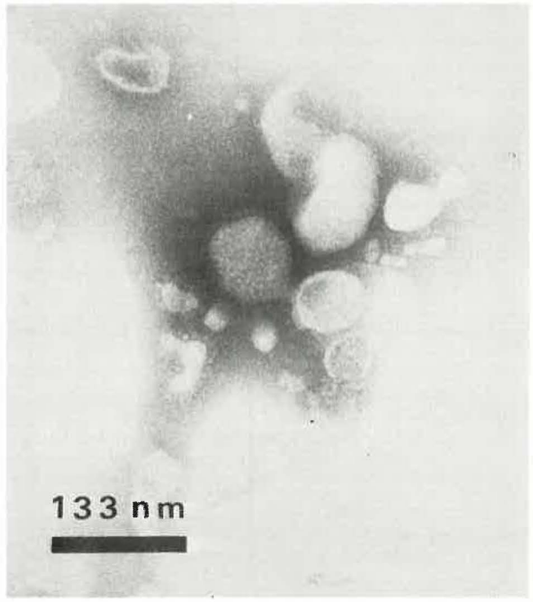

FIG. 3 Negatively stained virion purified from a lung rinse pellet of sheep 2283 by zonal centrifugation in a sucrose gradient

Serial transmission of jaagsiekte to lambs

All attempts to cultivate *in vitro* the retrovirus described above have been unsuccessful to date. However, the disease has been serially transmitted to new-born lambs over a number of years in several parallel experiments, 2 of which are illustrated in Fig. 4. Earlier transmissions were done with cell cultures and unpurified cell homogenates. For the last 3 serial passages in both experiments, however, progressively more purified preparations with progressively higher RDP activities were used. The results show a parallel decrease in the incubation period (lag) and a concomitant increase in the concentration of virus in the lung fluid, as measured by RDP activity (Fig. 4). The inocula used for the last 2 passages consisted of purified material, essentially homogeneous in the electron microscope (results not shown). Incubation periods were dramatically reduced to 3–5 weeks in the last experiments shown, presumably because of the increase in viral concentration in the lung rinse pellets used as inocula. This result can be regarded as strong evidence that the retrovirus described is in fact the aetiological agent of jaagsiekte.

Discussion

Previous claims for a viral aetiology in jaagsiekte was based mainly on circumstantial evidence such as transmission by contact and experimental transmission by means of lung secretion and tissue extracts (Tustin, 1969). Filtration experiments excluded organisms larger than viruses but not virus-like agents such as viroids or the scrapie agent.

After proving that jaagsiekte can be transmitted efficiently to experimental animals with a cell-free homogenate of cultured tumour cells (15,4) (Verwoerd *et al.*, 1980), we failed to demonstrate either virions or reverse transcriptase activity in either this

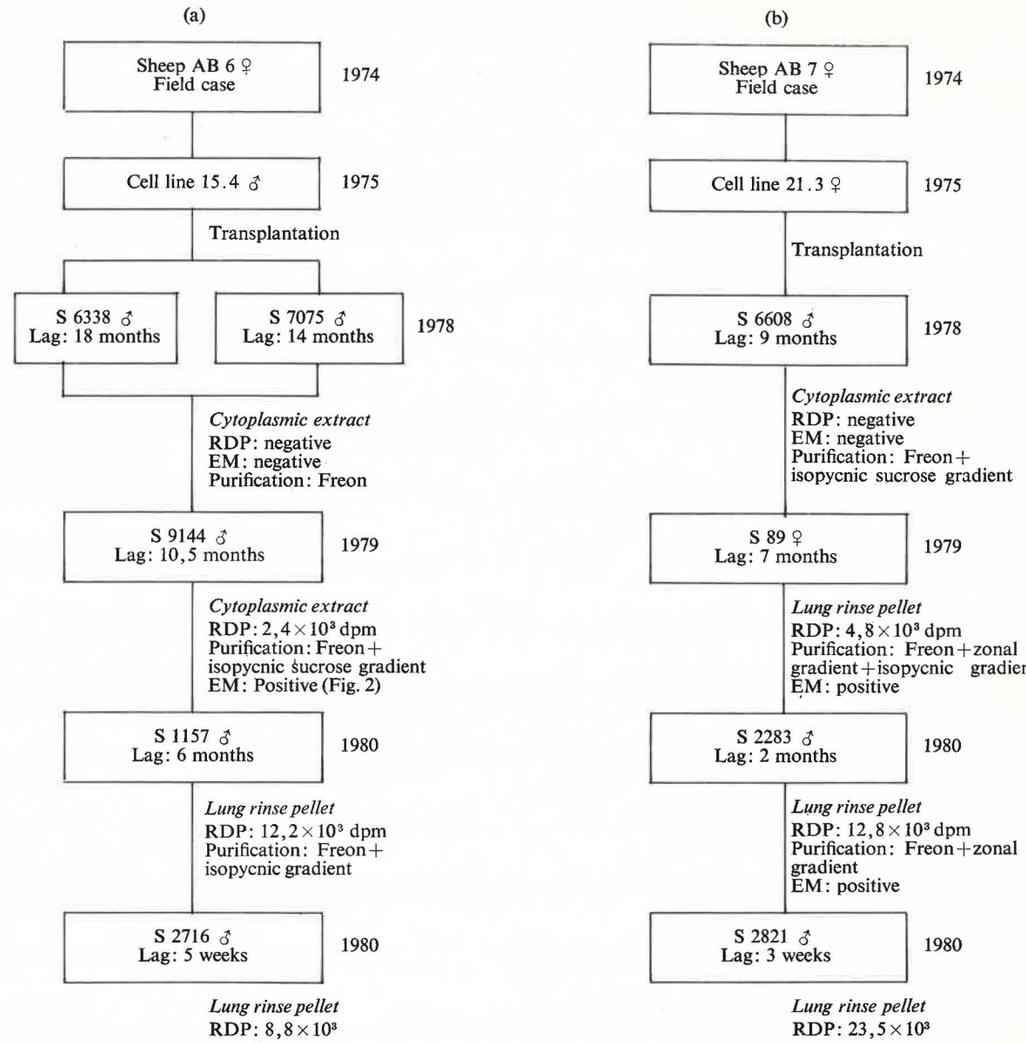

FIG. 4 Flow diagram of some serial transmissions of jaagsiekte to new-born lambs during recent years to illustrate the role of purified virus preparations

cell line or in lung homogenates. We therefore had to consider other possibilities such as defective or latent viruses, viroids and scrapie-like agents. When all attempts to rescue defective or latent retroviruses were unsuccessful, we proceeded to fractionate oncogenic cells and tissue homogenates and to test the various fractions for biological activity in new-born lambs. To date, this is the only assay system available, and, although very slow and costly, it was found to be quite dependable.

The results presented here (Tables 1 & 2) clearly indicate that oncogenicity resides in a cytoplasmic fraction with a density between 1,14 and 1,18.

This density range coincides with that of typical retroviruses (1,16–1,18), but again we found no RDP activity or virus particles in the biologically active density fraction. The scrapie agent, which is at present considered to be a small DNA molecule strongly attached to membranes (Malone *et al.*, 1979) also bands at this density and it is conceivable that viroids, consisting of free RNA (Diener, 1979) could also interact and band with cellular membranes in this density range. RNA and DNA were therefore isolated from both lung cytoplasmic fractions and 15.4 tumour cells, previously shown to be infective, using the techniques developed for these agents. No infectivity could be demonstrated (Table 2).

Various treatments of the oncogenic cytoplasmic extract were then used to further differentiate between the various possibilities. The crude scrapie agent is not inactivated by incubating at 80°C for 60 min nor by treatment with neutral detergents, fluorocarbons, nucleases or proteases. However, it is sensitive to treatment with aqueous phenol (Gibbons & Hunter, 1967; Malone *et al.*, 1979). Viroids, which are also relatively heat stable, are not inactivated by aqueous phenol or DNase treatment but are completely destroyed by treatment with RNase (Diener, 1979.) As reported in Table 3, the jaagsiekte agent is heat labile, sensitive to treatment with detergent or phenol but not to treatment with fluorocarbon or DNase. It is only partially inactivated by RNase and proteinase K. We conclude that it can neither be a scrapie-like agent nor a viroid. It possesses the characteristics

expected of an enveloped RNA-containing virus, which again suggests the possibility of its being a retrovirus.

Fluorocarbon extraction not only resulted in considerable purification of crude extracts without inactivating the agent, but it also seemed to enhance its infectivity. The first experimental case of jaagsiekte (Sheep 9144) produced with freon-purified lung extract (Fig. 4) yielded a lung extract in which we could demonstrate RDP activity for the first time. It also had a lung exudate in which a much higher enzyme activity was found, confirming the results of Sharp & Herring (personal communication). It is not clear why we did not find RDP activity in cases preceding Sheep 9144, whereas all subsequent cases consistently yielded positive results. Conceivably, the virus concentration in field cases and in our tumour cell line is so low that it cannot be detected by the RDP-assay, especially as we did not test lung exudates in the early cases.

It is evident that the concentration of virus in lung exudate, as measured by the RDP-assay, increased during serial passage in lambs (Fig. 4). This enabled us to concentrate and purify virus from lung rinse material, using fluorocarbon treatment followed by zonal and isopycnic centrifugation techniques in sucrose gradients. The buoyant density of 1,175 for the virion in sucrose determined in this way is similar to that reported for Type B and Type D retroviruses (Fine & Schochetman, 1978). Electron microscopy of the purified material revealed pleomorphic particles which vary in size between 75 and 110 nm and also in its apparent electron-density. The most consistent particles were about 85–95 nm in diameter and electron-dense, and had a close fitting membrane. Similar viral particles have been descrbied for Type D and Type B retroviruses (Fine & Schochetman, 1978). There can be little doubt, therefore, that a new retrovirus has been isolated and identified from jaagsiekte lungs.

The most important result from the transmission studies illustrated in Fig. 4, however, is the fact that virus preparations purified for density 1,175 and for the possession of maximal RDP-activity were highly efficient in producing cases of jaagsiekte and progressively reduced the incubation period before the appearance of symptoms from 12 months to 3 weeks in serial experiments. Although the virus has not yet been cultivated *in vitro* and Koch's postulates cannot therefore be fulfilled in the strict sense of the word, we regard these results as sufficient proof that the virus isolated is, in fact, the aetiological agent of jaagsiekte.

Finally, the question arises of a possible relationship between our virus and the ovine retrovirus causing maedi or progressive pneumonia. A direct serological comparison has not yet been made, but electron microscopically the 2 viruses are quite distinct. The following observations also indicate that the viruses are probably unrelated. Firstly, maedi does not occur in South Africa and a recent survey of a random selection of sheep sera, including some from jaagsiekte cases, demonstrated the complete absence of maedi antibodies (unpublished results). Secondly, all our experimentally produced jaagsiekte cases were examined histologically and maedi lesions were never seen, and, thirdly, attempts made to cultivate our isolate in sheep choroid plexus cells using the methods developed for visna-maedi virus were unsuccessful.

Further studies to characterize our virus more fully and to study its serological relationship to other retroviruses are being currently pursued.

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RESEARCH COMMUNICATION

A SURGICAL TECHNIQUE FOR THE EXPERIMENTAL REPRODUCTION OF EPIDIDYMITIS IN RAMS

B. C. JANSEN, SA Wool Board, P.O. Box 1378, Pretoria 0001

ABSTRACT

JANSEN, B. C., 1980. A surgical technique for the experimental reproduction of epididymitis in rams. *Onderstepoort Journal of Veterinary Research*, 47, 281–283 (1980).

A surgical technique is described for introducing a bacterial culture into the vas deferens of a ram close to the epididymis in such a manner that the infective material spreads to the lumen of the ductus epididymidis.

Résumé

UNE TECHNIQUE CHIRURGICALE POUR LA REPRODUCTION EXPÉRIMENTALE D'UNE ÉPIDIDYMITE CHEZ LE BÉLIER

Une technique chirurgicale pour l'introduction d'une culture de bactéries dans le vas diferens d'un bélier à proximité de l'épididyme, de telle manière que le matériel infectieux se propage dans le lumen du ductus épididymis, est décrite.

INTRODUCTION

An investigation into the aetiology and pathogenesis of ram epididymitis calls for the reproduction of the condition by a method which resembles the natural evolution of the disease. This implies that the infectious agent has to be introduced into the lumen of the ductus epididymidis to conform to the pathogenesis of epididymitis in nature as explained by Jansen (1980). He showed that bacteria from the environment entering the preputial cavity migrate up the genital canal in a direction retrograde to the flow of the semen, ultimately to land in the lumen of the ductus epididymidis. At this site they can initiate a pathological process.

Although attempts at causing orchitis and epididymitis by injecting cultures of *Actinobacillus seminis* or infected semen into the testes or epididymides of rams have been successful in causing an inflammation of these organs (Baynes & Simmons, 1960; Van Tonder, 1977), this method can hardly be regarded as a means of imitating the natural route of infection. The organisms would be deposited by this method in the interstitial tissue rather than in the lumen of the tubuli.

In the present study the author aimed at introducing bacterial cultures into the vas deferens in such a way that the organisms would reach the lumen of the ductus epididymidis.

MATERIALS AND METHODS

Rams with clinically normal genitalia as determined by inspection and palpation were selected. Furthermore, semen specimens were obtained by electro-ejaculation and only animals yielding semen from which no bacteria could be isolated were used in the experiments.

After 20 h without food and water, the rams were anaesthetized by a slow intravenous injection of a freshly prepared 10% solution of chloral hydrate until all palpebral reflexes disappeared. Then the scrotal sac was shaved and thoroughly cleaned with 70% ethyl alcohol.

The ram was placed on its back on the operating table with its head in a lower position than its body and with its mouth pointing downwards to avoid the inhalation of rumen fluid if it happened to be regurgitated.

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With the application of standard surgical techniques the posterior aspect of the scrotal sac was opened by a vertical incision on the midline at the level of the caput epididymidis. Subsequently, the tunica vaginalis of the left testis was reached by blunt dissection and opened by a vertical incision over the vas deferens which lies anterior to the body of the epididymis on the medial aspect of the testis. The vas deferens, which can be felt as a string-like structure, together with its blood-vessels, is contained in a fold. The identification of the vas deferens can be further facilitated if one closes it gently with one's fingers at the body end of the incision and has an assistant massage the semen from the cauda epididymidis into the lumen of the vas deferens.

The left vas deferens was selected to enable a right-handed surgeon to grasp the free end of the fold containing the vas deferens between the thumb and index finger of his left hand while the injection is done with the right hand. The right epididymis consequently served as a control.

A No. 26 Luer-Lock hypodermic needle attached to a 1 mℓ tuberculin syringe containing the infective material was gently introduced into the lumen of the vas deferens as close to the cauda epididymidis as possible. A volume of 0,1 mℓ of culture suspension containing about 1×10^8 organisms per mℓ was injected in a direction against the natural flow of the sperm cells.

To prevent leakage of the contents of the vas deferens through the puncture caused by the injection, a small area around the point where the needle was introduced was lightly cauterized with an electrocautery apparatus immediately after withdrawal of the needle and before release of the hold on the vas deferens.

The incision in the tunica vaginalis was then closed with interrupted stitches. The cavity left in the subcutaneous tissue between the testes was subsequently eliminated by appropriate stitching, and finally the skin was closed with interrupted stitches.

To establish whether a substance in suspension injected into the vas deferens at the site described does in fact spread to the lumen of the tubuli epididymidis, 0,1 mℓ of India ink was injected into the vas deferens of one of the rams. After 24 h the ram was killed and its epididymis examined histologically for the presence of carbon particles in the lumen of its tubules.