

## PRESENCE OF *HERPESVIRUS OVIS* DNA SEQUENCES IN CELLULAR DNA FROM SHEEP LUNGS AFFECTED WITH JAAGSIEKTE (PULMONARY ADENOMATOSIS)

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### ABSTRACT

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To investigate further the possible involvement of *Herpesvirus ovis* in the aetiology of jaagsiekte, the kinetics of reassociation of viral DNA and DNA isolated from tumour tissue as well as from cell cultures derived from it were studied. Although DNA-DNA hybridization could be demonstrated in 2 cases of jaagsiekte, no correlation was found between the presence of *Herpesvirus ovis* genome sequences and the occurrence of the disease.

### Résumé

PRÉSENCE DE SÉQUENCES DNA DU VIRUS HERPES OVIN DANS LES POUMONS DU MOUTON AFFECTÉ PAR L'ADÉNOMATOSE PULMONAIRE

Aux fins d'étudier le rôle éventuel du virus herpes ovin dans l'aétiologie de l'adenomatose pulmonaire, les cinétiques de la re-association de DNA virale et de DNA isolée de tissus de tumeur aussi bien que des cultures de cellules qui en dérivait ont été étudiées.

Bien que l'hybridation DNA-DNA put être démontrée dans deux cas d'adenomatose pulmonaire, aucune corrélation ne fut trouvée entre la présence de séquences des genome du virus herpes ovin et l'occurrence de la maladie.

### INTRODUCTION

The isolation of *Herpesvirus ovis* in various laboratories from jaagsiekte tissue or from cell cultures derived from it (Smith & Mackay, 1969; Malmquist, Krauss, Moulton & Wandera, 1972; Cvjetanovic, Forsek, Nevjestic & Rukanvia, 1972; De Villiers, Els & Verwoerd, 1975) suggested a possible involvement of this virus in the aetiology of the disease, and various attempts have been made to establish such a relationship between the two. A serological survey demonstrated that neutralizing antibodies against this virus are widely distributed throughout southern Africa (Verwoerd, Meyer-Scharrer, Broekman & De Villiers, 1979). Furthermore, the virus is closely related to or identical with an isolate made in Scotland, and therefore seems to be rather widely distributed. No correlation was found, however, between the neutralizing antibody titres and the presence or extent of jaagsiekte lesions (Verwoerd *et al.*, 1979).

Attempts to induce jaagsiekte by inoculating sheep with *Herpesvirus ovis*-infected cell cultures have failed (Mackay & Nisbet, 1972; Martin, Scott, Sharp, Angus & Norval, 1976; Martin, Angus, Robinson & Scott, 1979). In our laboratory a total of 48 lambs were inoculated with the virus in various combinations without producing a single case of the disease (De Villiers *et al.*, 1975; De Villiers, 1979a). Thus, neither serological nor transmission studies yielded evidence of the involvement of the virus in overt form, but the presence of a latent viral genome in transformed cells could not be excluded.

In the present communication we report attempts to demonstrate the presence of *Herpesvirus ovis* DNA sequences in the cellular DNA derived from adenomatous tumour cells by means of molecular hybridization techniques (Britten, Graham & Neufeldt, 1974).

### MATERIALS AND METHODS

#### *Purification of Herpesvirus ovis DNA*

*Herpesvirus ovis* was pelleted from the supernatant of infected cell cultures and its DNA isolated as described previously (De Villiers, 1979b). Briefly,

<sup>3</sup>H-labelled virus was disrupted by adding 2% Sarkosyl NL-97<sup>(1)</sup> and 1.0 mg/ml Proteinase K<sup>(2)</sup> and incubating it for 1 hour at 37 °C, and then for 1 hour at 60 °C (De Villiers, 1979b). It was then layered onto a 20-35% (w/w) sucrose gradient in a buffer containing 300 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 7.4) and 0.2% Sarkosyl NL-97 (Zur Hausen & Schulte-Holthausen, 1970). Sedimentation was carried out in a SW 41 rotor at 25 000 rpm for 16 hours at 20 °C. The gradient was fractionated and aliquots were precipitated with 5% trichloro-acetic acid (TCA) and 10 mM sodium pyrophosphate after addition of 20 µg/ml bovine serum albumin per fraction. Precipitates were collected on nitrocellulose filters which were dried and counted in a Beckman LS-9 000 scintillation counter after addition of toluene scintillation solution. The peak fractions were pooled and the DNA precipitated with 200 mM ammonium acetate and 2.5-3 volumes of ethanol for a few hours at -20 °C. Centrifugation followed for 50 minutes in a SW 41 rotor at 35 000 rpm at 0 °C. The precipitate was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

#### *Extraction of cellular DNA*

Small pieces of randomly selected tumour (about 5 g) were homogenized in 5 ml TE and incubated with 1% Sarkosyl NL-97 and 1 mg/ml pronase for 30 minutes at 37 °C. Subsequently, protein was removed by 2-3 extractions with equal volumes of phenol. Dialysis against TE buffer followed for 3 days. After incubation with 20 µg/ml RNase for 30 minutes, phenol extraction was again carried out and the phenol was removed by a further dialysis for 3 days. Aliquots of 500 µg were freeze-dried for use in the hybridization studies.

#### *Reference DNA*

Calf thymus DNA, fragmented by being passed repeatedly through a 20 gauge needle, was used as a negative control. Positive controls consisted of calf thymus DNA plus 1.0 genome equivalent per cell of unlabelled *Herpesvirus ovis* DNA as well as cellular

<sup>(1)</sup> Ciba-Geigy, Isando, RSA

<sup>(2)</sup> Merck Chemicals, Johannesburg, RSA



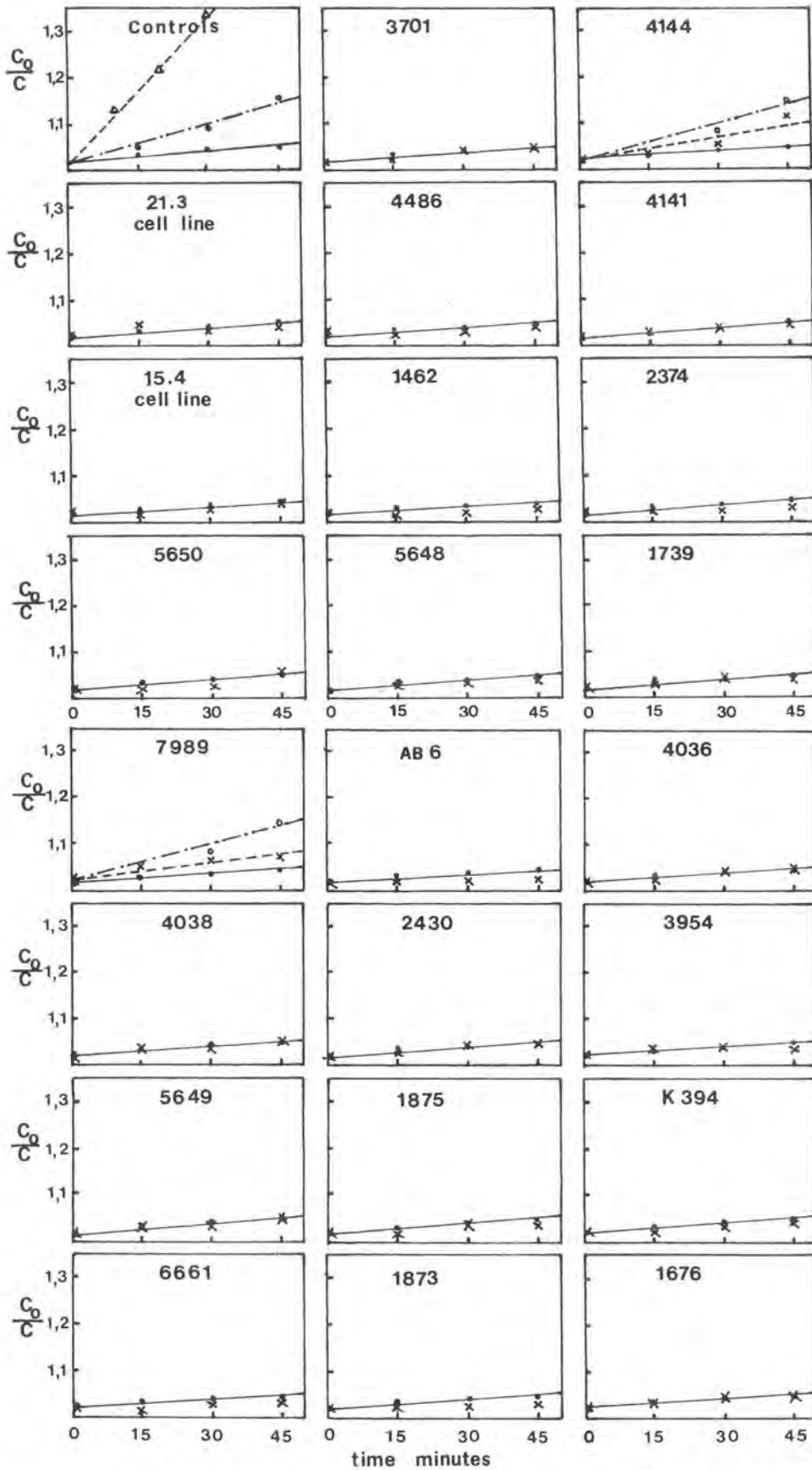


FIG. 1 Rates of reassociation between the radioactive DNA probe complementary to *Herpesvirus ovis* DNA and various DNA test samples

- △-----△ positive control: DNA from *H. ovis* infected lamb kidney cells
- negative control: calf thymus DNA
- positive control: calf thymus DNA plus one genome *H. ovis* DNA per cell.
- x-----x test sample: DNA from cell line indicated or lung tissue of the experimental animal identified by the number in each box.



DNA from *Herpesvirus ovis* infected FSK cells. For calculating the amounts of DNA used, the molecular mass of mammalian DNA was taken as  $3,9 \times 10^{12}$  (Lancaster, Olson & Meinke, 1977) and that of *Herpesvirus ovis* DNA as  $6,7 \times 10^7$  daltons (De Villiers, 1979b). One genome equivalent of viral DNA was calculated to be  $0,0172 \mu\text{g}$ .

*In vitro transcription*

Highly labelled DNA, complementary to the viral DNA, was synthesized *in vitro* in a nick-translation reaction for use as a hybridization probe. Between 0,1 and 1,0  $\mu\text{g}$  of viral DNA was nicked by incubation for 10 minutes at 17 °C with 6  $\mu\text{g}$  DNase in 250  $\mu\text{l}$  of a reaction mixture containing 50 mM Tris-HCl (pH 7,4), 5 mM MgCl<sub>2</sub>, 10 nanomoles each of dATP, dCTP and dGTP, and 200  $\mu\text{Ci}$  lyophilized <sup>3</sup>H-TTP (specific activity 46 Ci/mmol). DNA polymerase I (Kornberg enzyme) was then added at a rate of 5 units (1 ml) per 0,2  $\mu\text{g}$  viral DNA, and the mixture incubated for a further 60 minutes at 17 °C. Synthesis was terminated by extraction of the protein with an equal volume of phenol (saturated in TE, pH 8,1). Non-incorporated isotope was removed by passing the sample through a Sephadex G-50 column, eluting with 20 mM Tris-HCl and 2 mM EDTA (pH 7,4). Aliquots of 5  $\mu\text{l}$ /fraction were counted in a toluene-Triton-X-100 scintillation mixture in a Beckman LS-9 000 scintillation counter. The peak fractions containing the labelled viral DNA were pooled and stored at -20 °C after the radio-activity was measured. Sedimentation analysis of this labelled DNA was carried out as described by De Villiers (1979b).

*Determination of reassociation kinetics*

Samples of 500  $\mu\text{g}$  of lyophilized cellular DNA were dissolved in water, 14  $\mu\text{g}$  of tritium labelled viral DNA (0,8 genome equivalent,  $1,29 \times 10^5$  cpm) added, and the buffer concentration adjusted to 100 mM Tris-HCl and 10 mM EDTA (pH 7,4). The DNA was denatured in an ethylene-glycol bath at 104 °C for 15 minutes and then adjusted to final concentrations of 350 mM NaCl, 12 mM EDTA, 0,06% sodium dodecyl sulphate (SDS) and 100 mM sodium-phosphate buffer (pH 6,8) at 4 °C.

The aliquots were then chromatographed on 1 ml hydroxyapatite columns maintained at 60 °C. Single-stranded DNA was eluted first with 2 ml of 140 mM sodium phosphate buffer (pH 6,8) containing 0,4% SDS and then with 1 ml of a 180 mM sodium phosphate buffer (pH 6,8) containing 0,4% SDS. Double-stranded DNA was eluted with 1,5 ml 400 mM sodium phosphate buffer (pH 6,8) containing 0,4% SDS. Eluates were adjusted to identical phosphate and SDS concentrations in a total volume of 4 ml and counted in a toluene-Triton X-100 scintillation mixture in the gel form at 4 °C (Fleckenstein, Bornkamm & Ludwig, 1975). Reassociation rates were calculated according to Wetmur & Davidson (1968).

RESULTS

*In vitro transcription product*

A specific activity of  $7,4-11,7 \times 10^6$  cpm per  $\mu\text{g}$  DNA input was obtained for the complementary DNA probe synthesized *in vitro*. Sedimentation analysis on sucrose gradients (results not shown) yielded a sedimentation constant of 4S for the probe, compared to 7S to 8S for the cellular DNA and the calf thymus DNA.

*Reassociation kinetics*

The reassociation of DNA follows the kinetics of a second-order reaction which can be expressed as  $C/C_0 = \frac{1}{1+KC_0t}$ , where C<sub>0</sub> represents the concentration of single-stranded DNA (measured as cpm) at time zero and C the concentration at time t. If C/C<sub>0</sub> is plotted against t, the slope of the resulting line is the reassociation constant K, which is a measure of the rate of reassociation.

The reassociation rates of the various DNA samples which were tested are shown in Fig. 1. The slopes shown in the first (top left) rectangle indicate that the reassociation reaction between the probe and DNA from virus-infected cells ( $\Delta$ ----- $\Delta$ ) proceeded 12,2 times faster than the reaction with calf thymus DNA ( $\bullet$ ----- $\bullet$ ), whereas the reassociation rate with 1 genome equivalent unlabelled viral DNA ( $\circ$ ----- $\circ$ ) is 2,3 times that of the negative control DNA.

In each of the other rectangles the reassociation rate of a DNA test sample ( $\times$ ----- $\times$ ) is compared with that of the negative control ( $\bullet$ ----- $\bullet$ ). No increase was found in the reassociation rates of DNA isolated from cell cultures of 21.3 and 15.4, two tumour cell lines established from jaagsiekte lungs, reflecting the absence of sequences homologous to the viral DNA in the tumour cell DNA. Similarly, all 7 DNA samples from normal sheep lungs (5650, 3701, 4486, 5648, 1739, 5649 and 1676) gave negative results. An increase in the reassociation rate above that of the control was detected in only 2 of the 14 samples isolated from jaagsiekte lesions: DNA from Sheep 4144 reassociated twice as fast and from Sheep 7989 1,5 times faster than the control. By comparing these rates with those obtained in the reaction with 1 genome of viral DNA, it was calculated that these two lungs contained on average 0,87 and 0,65 viral genomes per cell.

DISCUSSION

The jaagsiekte lung tumours tested for the presence of *Herpesvirus ovis* genetic material by means of reassociation kinetics were a random sample of the tumours available. Five sheep (7989, AB6, 1873, 1875 and 394) were field cases of jaagsiekte; the others were experimentally produced. The latter group included animals in which the tumours were produced by transplantation of the 15.4 cell line (1462, 4144, 4141, 2374, 2430, 4036, 4038 and 3954) or by injection of a cytoplasmic extract (6661). Only 3 of the 14 animals possessed neutralizing antibodies against *Herpesvirus ovis*. No correlation was found between any of these factors and the 2 cases in which viral DNA was present in the cell genome. Sheep 4144 (which had no antibodies against the virus) was a case produced experimentally by injecting 15.4 tumour cells. In contrast to Sheep 4144, Sheep 7989 was a field case possessing neutralizing antibodies.

These results bear little resemblance to those obtained in the case of the Epstein-Barr human herpesvirus, where viral DNA was consistently found in the tumour cells or lymphocytes of the majority of cases of Burkitt's lymphoma, nasopharyngeal carcinoma and infectious mononucleosis (Zur Hausen, 1975). The majority of jaagsiekte lesions appear to be negative for the presence of *Herpesvirus ovis* DNA. We conclude that those cases where evidence for the presence of viral genomes was found represent latent infections of the virus which has been shown to be almost ubiquitous in its distribution (Verwoerd *et al*, 1979).



Both cell lines tested were established from jaagsiekte lesions and produced typical jaagsiekte lesions when injected intratracheally into lambs (Verwoerd, De Villiers & Tustin, 1980). The absence of viral sequences from the genomes of these cells seems to exclude any role for the virus in the aetiology of jaagsiekte, with the possible exception of a helper function or a role during initial transformation only.

REFERENCES

BRITTEN, R. J., GRAHAM, D. E. & NEUFELD, B. R., 1974. Analysis of repeating DNA sequences by reassociation. In: GROSSMAN, L. & MOLDAVE, K. (Ed.), Vol. 29, Part E, 363-418. New York and London: Academic Press.

CVJETANOVIC, V., FORSEK, Z., NEVJESTIC, A. & RUKANVIA, Lj., 1972. Isolation of the sheep pulmonary adenomatosis (SPA) virus. *Veterinaria*, 21, 493-496.

DE VILLIERS, ETHEL-MICHELE, 1979a. *Herpesvirus ovis* and the aetiology of jaagsiekte. D.Sc. Thesis, University of Pretoria.

DE VILLIERS, E-M., 1979b. Purification of the JS-3 isolate of *Herpesvirus ovis* (Bovine Herpesvirus 4) and some properties of its DNA. *Journal of Virology*, 32, 705-709.

DE VILLIERS, ETHEL-MICHELE, ELS, H. J. & VERWOERD, D. W., 1975. Characteristics of an ovine herpesvirus associated with pulmonary adenomatosis (jaagsiekte) in sheep. *South African Journal of Medical Sciences*, 40, 165-175.

FLECKENSTEIN, B., BORNKAMM, G. W. & LUDWIG, H., 1975. Repetitive sequences in complete and defective genomes of *Herpesvirus saimiri*. *Journal of Virology*, 15, 398-406.

LANCASTER, W. D., OLSON, C. & MEINKE, W., 1977. Bovine papilloma virus: Presence of virus-specific DNA sequences in naturally occurring equine tumours. *Proceedings of the National Academy of Sciences*, 74, 524-528.

MACKAY, J. M. K. & NISBET, D. I., 1972. Pathogenicity tests in lambs with an ovine herpesvirus. In: BIGGS, P. M., DE-THE & DAYNE, L. N. (Ed.), pp. 467-468. IARC Scientific Publications No. 2, Lyon.

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

MARTIN, W. B., ANGUS, K. W., ROBINSON, G. W. & SCOTT, F. M. M., 1979. The herpesvirus of sheep pulmonary adenomatosis. *Comparative Immunology, Microbiology and Infectious Diseases*, 2, 313-325.

MARTIN, W. B., SCOTT, F. M. M., SHARP, J. M., ANGUS, K. W. & NORVAL, M., 1976. Experimental production of sheep pulmonary adenomatosis (jaagsiekte). *Nature, London*, 264, 183-185.

SMITH, W. & MACKAY, J. M. K., 1969. Morphological observations on a virus associated with sheep pulmonary adenomatosis (jaagsiekte). *Journal of Comparative Pathology*, 79, 421-427.

VERWOERD, D. W., DE VILLIERS, ETHEL-MICHELE & TUSTIN, R. C., 1980. Aetiology of jaagsiekte: experimental transmission to lambs by means of cultured cells and cell homogenates. *Onderstepoort Journal of Veterinary Research*, 47, 13-18.

VERWOERD, D. W., MEYER-SCHARRER, ELIZABETH, BROEKMAN, J. & DE VILLIERS, ETHEL-MICHELE, 1979. The serological relationship of *Herpesvirus ovis* to other herpesviruses and its possible involvement in the aetiology of jaagsiekte. *Onderstepoort Journal of Veterinary Research*, 46, 61-63.

WETMUR, J. G. & DAVIDSON, N., 1968. Kinetics of renaturation of DNA. *Journal of Molecular Biology*, 31, 349-370.

ZUR HAUSEN, H., 1975. Oncogenic herpesviruses. *Biochimica et Biophysica Acta*, 417, 25-53.

ZUR HAUSEN, H. & SCHULTE-HOLTHAUSEN, H., 1970. Presence of EB virus nucleic acid homology in a "virus-free" line of Burkitt tumour cells. *Nature, London*, 227, 245-248.