Epithelial tumour cells in the lungs of sheep with pulmonary adenomatosis are major sites of replication for Jaagsiekte retrovirus

M. Palmarini, P. Dewar, M. De las Heras, N. F. Inglis, R. G. Dalziel and J. M. Sharp

Sheep pulmonary adenomatosis (SPA) is a naturally occurring contagious lung tumour of sheep which has been associated aetiologically with a type D- and B-related retrovirus (Jaagsiekte retrovirus; JSRV). To improve understanding of the aetio-pathogenesis of SPA, the distribution and the sites of JSRV replication in sheep with naturally or experimentally induced SPA or in unaffected controls were identified. New immunological reagents were produced and a blocking enzyme-linked immunosorbent assay (B-ELISA) and an immunohistochemical technique for the detection of JSRV major capsid protein at the tissue and cellular levels were developed. JSRV was detected only in the respiratory tract of sheep affected by pulmonary adenomatosis and specifically in the transformed epithelial cells of the alveoli of SPA-affected sheep.

Introduction

Retroviruses related to the type D viruses of primates have been demonstrated in the house shrew (Tsutsui et al., 1985; Aoyama et al., 1988), sheep (Sharp & Herring, 1983; He et al., 1992; De las Heras et al., 1993), cattle (York et al., 1989) and goats (De las Heras et al., 1991). Of these type D viruses, only the simian retroviruses and the small ruminant retroviruses have been associated with any disease (Marx & Lowenstine, 1987; Sharp, 1987). The small ruminant viruses have been associated with three contagious tumours, sheep pulmonary adenomatosis (syn. SPA, jaagsiekte, ovine pulmonary carcinoma) (Sharp & Angus, 1990), enzootic nasal tumour of sheep (De las Heras et al., 1993) and enzootic nasal tumour of goats (De las Heras et al., 1991), all of which arise from secretory epithelial cells in the respiratory tract.

Each of these tumours has been shown consistently to contain a retrovirus with morphological, biochemical and immunological properties similar to type D retroviruses and cell-free tumour extracts can be used to reproduce these diseases experimentally (Cohrs, 1953; Martin et al., 1976; Verwoerd et al., 1980; Sharp et al., 1983; DeMartini et al., 1988; De las Heras et al., 1995).

Further, the incubation period of experimentally induced sheep pulmonary adenomatosis (SPA) can be reduced to a few weeks or days (Verwoerd et al., 1980; Sharp et al., 1983) and has been shown to be inversely related to the reverse transcriptase activity in the inoculum (Verwoerd et al., 1985). Although several lines of evidence implicate a retrovirus, known as Jaagsiekte retrovirus (JSRV) in the aetiology of SPA (reviewed by Sharp, 1987), obtaining definitive proof by classical approaches has been impeded by the absence of a cell culture system to propagate JSRV and a lack of reagents and techniques to undertake studies on the pathogenesis of the disease. Recently, one strain of JSRV has been cloned and the full-length genomic sequence determined (York et al., 1991, 1992). This virus exhibits the typical genomic organization of a replication-competent type D retrovirus with LTR, gag, pro, pol and env regions; there are no apparent sequences commonly associated with transformation (York et al., 1992). Southern hybridization has revealed that numerous related sequences are present in the genomes of both unaffected sheep and goats (York et al., 1992; Hecht et al., 1994), which raises the question of whether JSRV is involved in the aetiology of SPA or is an endogenous homologue reactivated during the neoplastic process. The availability of one sequence of JSRV permitted new approaches in the investigation of the aetio-pathogenesis of SPA. Studies were undertaken, therefore, to examine the distribution and sites of replication of JSRV in SPA-affected and unaffected sheep.
sheep to better define the role of type D retroviruses in the aetiology of SPA.

**Methods**

**Subcloning and expression of clone Js382.** Standard molecular biology procedures were used as described by Sambrook et al. (1989) and will not be reported in detail. Plasmid pBluescript-Js382 containing a part of the JSRV gag gene (bases 953 to 3030 of the nucleotide sequence published by York et al., 1992) was a gift from G. Quérat (Marseille, France). Briefly, the insert fragment was excised by EcoRI, subcloned into plasmids pMS1S (Sherf et al., 1990; supplied by M. Shreiber) and pGEX11T (Pharmacia) and expressed in E. coli host strain NM522 as β-galactosidase (βgal-CA, plasmid pMCA) and glutathione S-transferase (GST-CA, plasmid pGCA) fusion proteins respectively.

Confirmation that the gag gene was in the correct reading frame was obtained by sequencing across the vector–insert junction, as well as testing clones for production of β-galactosidase and GST fusion proteins of the appropriate size by Western blotting (immunoblot) analysis with a goat antisera to Mason–Pfizer monkey virus major capsid protein (MPMV-CA) (ref. 7SS-148, National Cancer Institute Repository) (Sharp & Herring, 1983).

Transformed bacteria were grown and induced with isopropyl β-D-thiogalactopyranoside (IPTG) for the expression of recombinant proteins. Bacteria were pelleted (5000 g for 10 min) and resuspended in 20 ml TE [10 mM-Tris pH 7.5, 1 mM-EDTA]. Phenylmethylsulphonyl fluoride (2 mM) was added before lysing the cell suspension in a French press at 1500 p.s.i. [10.35 MPa]. The lysate obtained was sonicated and clarified at 4 °C for 10 min.

βgal-CA fusion protein was purified by affinity chromatography using a 4 ml column of aminobenzyl 1-thio-fl-galactopyranoside (ABTG) agarose (Sigma) as previously described (Cameron et al., 1994). GST-CA was purified by affinity chromatography using a 4 ml column of glutathione-Sepharose (Pharmacia) as recommended by the manufacturers.

The yield of soluble βgal-CA was approximately 9 mg/1 of bacterial culture at about 75 % purity as estimated by SDS-PAGE. GST-CA could not be eluted with free glutathione from the Sepharose beads as recommended by the manufacturers and was therefore further used coupled to the beads to immunize rabbits.

**Production of rabbit polyclonal antisera to JSRV-CA.** A specific rabbit antisera to JSRV-CA was prepared by immunizing rabbits with 500 μg βgal-CA combined with Freund’s incomplete adjuvant. After 15 days the rabbit was boosted with 500 μg of GST-CA bound to the glutathione-Sepharose beads. A third injection of GST-CA (500 μg) was given after 4 weeks and the rabbit was bled 15 days after the last injection.

To avoid non-specific reactions in the B-ELISA and in the immunohistochemical study, the antisera obtained was absorbed overnight at 4 °C with a lysate of IPTG-induced NM522(pMS1S) cells. The serum was then centrifuged at 10000 g for 30 min to pellet any bacterial debris, aliquoted and stored at −20 °C until use.

By Western blotting the rabbit antiserum recognized the two recombinant proteins βgal-CA and GST-CA, as well as the native 25 kDa CA in JSRV. There was no reaction with any other protein in sucrose gradient purified JSRV (data not shown).

**Sources and preparation of samples.** In this study, SPA sheep were defined as animals which showed typical clinical signs, particularly the production of an abundant sero-mucoid fluid (lung fluid) from the nostrils when the rear limbs were elevated above the head, and confirmed by macroscopic and histological examination of the lungs.

To determine the best conditions for the B-ELISA and to assess its specificity and its inter- and intra-assay variability the following samples were employed: (a) two different pools of lung fluid (LFP#1 and 2) collected from several SPA sheep (in these samples the presence of JSRV was confirmed by Western blotting); (b) sucrose gradient purified maedi-visna virus (MVV); (c) a lysate of NM523(pMS1S) cells induced with IPTG to provide a β-galactosidase control; (d) a lysate of NM522(pGEX1T) cells induced with IPTG to provide a GST control. LFP#1 was filtered through a double layer of gauze and clarified by centrifugation at 100000 g for 1 h at 4 °C. The resultant supernatant was aliquoted and stored at −70 °C until use.

JSRV was extracted from LFP#2 as described by Herring et al. (1983). Briefly, LFP#2 was filtered, clarified and the supernatant was then centrifuged at 100000 g through a double layer of glycerol (25/50 %, v/v) for 1 h at 4 °C. An aliquot of the supernatant was stored at −70 °C before and after the ultracentrifugation step. The resultant pellet was resuspended in TNE buffer (100 mM-NaCl, 1 mm-Tris, 1 mM-EDTA) and further purified by isopycnic centrifugation on 20 to 55 % (w/w) sucrose gradients. The gradient was fractionated and each 0.5 ml fraction was resuspended in 4.5 ml TNE buffer, centrifuged at 100000 g for 1 h at 4 °C and the resultant pellet resuspended in 100 μl TNE.

For analysis of the distribution of JSRV in affected and unaffected animals, samples were collected from: (a) nine sheep naturally affected by SPA; (b) 16 lambs, experimentally infected as previously described (Sharp et al., 1983); (c) 16 age and breed-matched controls.

Samples of lung, lung tumour, mediastinal and retropharyngeal lymph nodes, thymus, tonsils, Peyser’s patches, spleen, bone marrow and kidney were collected during the post-mortem examination or immediately before the sheep were killed (lung fluid and heparinized blood samples). Lung fluid samples were filtered, clarified, at 10000 g for 1 h at 4 °C and the resultant supernatants were stored at −70 °C. Plasma samples (8 ml per sample) were further centrifuged at 100000 g for 1 h at 4 °C and the pellets resuspended in 65 μl of TNE buffer. Plasma samples were stored at −70 °C. Leukocytes were obtained from blood samples in which the red cells had been lysed. Each leukocyte sample (7 x 107 cells) was diluted in TNE, frozen/thawed three times, and clarified and centrifuged as described above. After the ultracentrifugation step, pellets were resuspended in 65 μl of TNE and stored at −70 °C.

Tissues samples were homogenized (10 % (w/v) suspension in TNE), filtered, clarified and centrifuged as above. All tissue samples were concentrated 400 times except tonsils which were concentrated only 100 times due to the small size of this organ. Tissues extracts were kept at −70 °C until further use.

**Blocking enzyme-linked immunosorbent assay (B-ELISA).** The SPA B-ELISA (Engvall & Perlmann, 1971) depends on the presence of JSRV in the test samples to inhibit the binding between purified βgal-CA and the rabbit antisera to JSRV-CA.

Equal volumes of the test sample and washing fluid B [WFB; PBS, 0.5 % (w/v), Tween 20, 1 mM-EDTA and 350 mM-NaCl] were reacted at room temperature for 1 h in order to disrupt the envelope of JSRV and release the capsid of the virions. WFB was also used as washing buffer for the ELISA plates and as dilution buffer for the reagents of the reaction. The optimal concentrations of βgal-CA and the rabbit antisera were determined before use. Microtitre plates were coated with 100 μl of purified βgal-CA (1.5 μg/ml), diluted in carbonate buffer (pH 9.6), by absorbing overnight at 4 °C in a humid chamber. After incubation, the plates were washed with WFB and blocked by incubation for 1 h at 37 °C with a 1 % (w/v) solution of bovine serum albumin in PBS.

The antisera and test samples were reacted first in a V-bottom 'transfer' plate (65 μl of 1:800 dilution of rabbit antisera to JSRV-CA + 65 μl of test samples pre-treated with WFB) for 1 h at 37 °C.
Thereafter, 100 μl of the resulting mixture (rabbit serum + sample) was transferred to the pre-coated ELISA plates, which had been washed three times with WFB.

The ELISA plates were incubated at 37 °C for 1 h and, after washing, 100 μl of pig antiserum to rabbit IgG conjugated to horse-radish peroxidase (DAKO; 1:1000 in WFB) was added to each well. After incubation at 37 °C for 1 h, the plates were washed again with WFB and 100 μl of substrate (o-phenyldiamine; Sigma) + 0.05 % H2O2 (30 %, v/v) was added to each well. The colour development was stopped with 2.5 m H2SO4 and the plates read using a Dynatech MR5000 apparatus at 490 nm.

Samples were tested in duplicate in each test. Controls included wells with no inhibitor (0 % blocking), and wells with neither inhibitor nor serum (100 % blocking), the missing component being replaced with diluent (WFB).

Results are expressed as a percentage blocking value calculated using the following formula

\[
\% \text{ blocking value} = \left( \frac{OD_{00} - OD_{test}}{OD_{00} - OD_{x00}} \right) \times 100
\]

where \(OD_0\) is the OD at 0 % blocking control, \(OD_{100}\) is the OD at 100 % blocking control and \(OD_{test}\) is the OD of the test sample.

The intra-assay standard deviation was evaluated testing LFP#1 across the ELISA plate in five different plates and calculating the mean between the standard deviations obtained in each plate. The inter-assay standard deviation was calculated assaying LFP#1 in ten independent tests performed on different days. LFP#1 was located in the same wells on the ELISA plate on each occasion.

**Western blotting.** Western blotting was performed on the purified βgal-CA and GST-CA and on LFP#1 sucrose gradient purified fractions employing goat antiserum to MPMV-CA and the rabbit antiserum to JSRV-CA as described previously (Sharp & Herring, 1983).

**Immunohistochemistry.** Tissue samples used in immunohistochemistry studies were collected during the post-mortem examination from four SPA-affected sheep (three experimentally infected lambs and one natural case of SPA) and three uninfected controls. The same tissues as collected for the B-ELISA were fixed in 10 % neutral buffered formalin, processed routinely in an automatic tissue processor, embedded in paraffin wax and sectioned at 4-6 μm. Selected sections were stained immunohistochemically using a commercial avidin-biotin peroxidase complex kit (Vectastain ABC kit; Vector Laboratories) as recommended by the manufacturers except that sections were treated additionally with 0.1 % (w/v) trypsin in TBS for 30 min at 37 °C. The rabbit antiserum to JSRV-CA was used as primary antibody at the predetermined optimal dilution of 1:50. This serum was substituted with TBS to control for the endogenous peroxidase activity of the tissue or with rabbit pre-immunization serum to check for non-specific reactions. Carazzi's haematoxylin was used as a counterstain.

**Results**

**Development and validation of the B-ELISA**

A series of experiments was performed to demonstrate that the blocking values obtained in the B-ELISA were the result of the interaction between JSRV-CA and the rabbit antiserum produced in this study and not due to non-specific reactions.

The analysis of the 18 fractions of LFP#2 separated by isopycnic centrifugation showed that the greatest %blocking values corresponded to fractions having a buoyant density between 1.16 g/ml to 1.19 g/ml, with a peak at 1.18 g/ml which is the buoyant density of JSRV (Sharp & Herring, 1983) (Fig. 1). The same fractions analysed by Western blotting, employing a goat antiserum to MPMV-CA and the rabbit serum produced in this study showed a single polypeptide with an M_r of about 25000, the predicted M_r of JSRV-CA.

In LFP#2 the blocking value before ultracentrifugation was 63 % which was reduced to 10 % for the post-centrifugation supernatant demonstrating that the blocking activity was particle-associated, and did not arise as a result of antibodies specific for JSRV-CA that might have been present in the lung fluid. In other experiments maedi-visna virus, another retrovirus of sheep which is occasionally found to coinfect SPA-affected animals (Rosadio et al., 1988a, b), the β-galactosidase control and the GST control gave %blocking values less than 7.

These data demonstrate that the blocking value of the SPA B-ELISA depends on the presence in the test samples of the major capsid protein (CA) of JSRV.

The intra-assay standard deviation was +1.6 % while the inter-assay standard deviation was 4.6 %.

**Distribution of JSRV viral particles in tissue extracts of SPA-affected and unaffected sheep**

Following the successful development of the B-ELISA to detect JSRV-CA, this assay was used to examine the anatomical distribution of JSRV in sheep. The samples
Table 1. Results of the B-ELISA on tissues of SPA affected and unaffected sheep

<table>
<thead>
<tr>
<th>Sample</th>
<th>SPA + * % blocking value (±SE)</th>
<th>n</th>
<th>SPA - † % blocking value (±SE)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung tumour/lung</td>
<td>77.8 (±4.8)§</td>
<td>13</td>
<td>17 (±1.8)</td>
<td>13</td>
</tr>
<tr>
<td>Lung fluid</td>
<td>68.4 (±3.3)</td>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maediastinal lymph nodes</td>
<td>15.2 (±3.7)</td>
<td>12</td>
<td>6.2 (±2.1)</td>
<td>8</td>
</tr>
<tr>
<td>Retropharyngeal lymph nodes</td>
<td>6.6 (±1.4)</td>
<td>8</td>
<td>7.0 (±2.4)</td>
<td>7</td>
</tr>
<tr>
<td>Tonsils</td>
<td>4.7 (±2.3)</td>
<td>9</td>
<td>3.7 (±1.5)</td>
<td>9</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>40.2 (±4.06)</td>
<td>6</td>
<td>45.2 (±5.7)</td>
<td>6</td>
</tr>
<tr>
<td>Thymus</td>
<td>18.7 (±3.8)</td>
<td>7</td>
<td>13.1 (±2.7)</td>
<td>13</td>
</tr>
<tr>
<td>Peyer's patches</td>
<td>12.6 (±1.1)</td>
<td>5</td>
<td>7.8 (±2.7)</td>
<td>6</td>
</tr>
<tr>
<td>Spleen</td>
<td>18.7 (±2.7)</td>
<td>9</td>
<td>16.1 (±3.9)</td>
<td>8</td>
</tr>
<tr>
<td>Kidney</td>
<td>6.5 (±2.9)</td>
<td>8</td>
<td>8.3 (±4.1)</td>
<td>7</td>
</tr>
<tr>
<td>Plasma</td>
<td>10.8 (±3)</td>
<td>10</td>
<td>7.6 (±3.1)</td>
<td>5</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>12.4 (±2.2)</td>
<td>10</td>
<td>13.4 (±1.1)</td>
<td>5</td>
</tr>
</tbody>
</table>

* SPA-affected animals.
† Unaffected controls.
‡ Number of samples tested.
§ Standard error of the mean.

Fig. 2. (a, b) Immunohistochemistry of a lung section of a sheep affected by SPA (bars, 40 μm; magnification 250 x ). The dark staining represents the positive reactions and is confined to the cytoplasm of the transformed cells of the adenoma. No staining is apparent in the extracellular airway spaces. A papillary projection of the tumour in the bronchiolus is visible in (b). The dark staining is confined to the neoplastic cells. No staining is observed in the interstitial cells or in the untransformed alveolar cells and bronchiolar cells.

tested were obtained by ultracentrifugation and therefore reflected the presence of JSRV viral particles. Statistical analysis of the differences between SPA-affected and unaffected animals was undertaken using Student's t-test. Results are summarized in Table 1.

Only tumour and lung samples showed a significant difference between affected and unaffected sheep (P < 0.00001). The mean % blocking value of tumour samples was 77.8 with values ranging from 41 to 93 while normal lung samples had a mean % blocking value of 17 with a range from 0 to 33.

A % blocking value of 36 was selected as the highest negative value calculated from the mean blocking value of control lungs plus 2.5 standard deviations. When this value was used no false positive or false negative samples were observed.

No significant difference was observed between the mean blocking values obtained using lung tumour samples collected from natural or from experimentally induced cases of SPA. Lung fluid samples from SPA cases showed a high % blocking value (mean = 68.4) with values ranging from 49 to 92 but because healthy sheep do not produce collectable quantities of lung fluid no direct control for this sample could be obtained. The closest control for the lung fluid samples was bronchoalveolar lavages from unaffected sheep, in which
% blocking values were always under 18 (data not shown).

In all the other tissues examined there was no statistically significant difference between SPA-affected and unaffected sheep with the exception of a single maediastinal lymph node sample, collected from a natural case of SPA. This sample had a % blocking value of 53, markedly greater than the mean % blocking value of equivalent tissues in control animals (6·25).

Bone marrow samples showed a high mean blocking value in both affected and unaffected controls. The mean % blocking values were 40·2 for SPA sheep and 45·2 for the unaffected controls. These high % blocking values are probably an artefact due to the nature of the sample after processing. The preparation of this tissue as described in Methods resulted in a viscous pellet which therefore could have blocked the reaction non-specifically. Further dilutions or sucrose gradient purified samples resulted in the almost complete loss of blocking activity and no specific reaction in this tissue was found by immunohistochemistry (see below) or Western blotting.

Cellular localization of JSRV-CA

Immunohistochemical analysis showed that JSRV major capsid protein was detected in the cytoplasm of recognizable alveolar neoplastic cells in all four SPA lungs examined (Fig. 2a, b). There was no staining of the epithelial non-transformed cells nor of the interstitial cells. Specific staining in the extracellular airway spaces was observed very rarely. No specific staining was detected in any other tissue.

Discussion

The results of this study using immunological techniques have shown that JSRV virions and CA were detected only in the respiratory tract of sheep affected by SPA. These findings have confirmed earlier reports describing type D retrovirus antigens or reverse transcriptase activity in respiratory tissues of SPA sheep (Perk et al., 1974; Martin et al., 1976; Herring et al., 1983; Sharp & Herring, 1983; Verwoerd et al., 1983; Rosadio et al., 1988a) and extended these observations by demonstrating that JSRV was not detected in 172 samples from eight other tissues from 41 sheep, although viral expression, below the detection limit of the B-ELISA, may be occurring in other tissues.

Immunohistochemistry supported the above conclusion and further indicated that JSRV replication occurred primarily in the transformed epithelial cells in the pulmonary alveoli of SPA sheep. A notable exception was the positive result obtained with a single lymph node from a field case of SPA that probably arose as a consequence of metastatic events, which are known to occur in a proportion of cases (Nobel et al., 1969). Although immunohistochemical studies employing a heterologous antiserum to MPMV-CA detected putative JSRV antigens in the alveolar lumina, rather than intracellularly (Payne et al., 1986), and therefore conflict with those reported here, the observations of the present communication, obtained with an homologous antiserum, are in agreement with earlier ultrastructural studies describing intracytoplasmic type A particles in the epithelial tumour cells and, associated with these cells, a few extracellular particles with typical type D morphology (Perk et al., 1971; Sharp et al., 1983; Payne et al., 1983).

The above findings point to the epithelial tumour cells in the alveoli as the major site of translation and assembly of JSRV and highlight the strict association between JSRV and the tumour. These findings, therefore, provide further support for the notion that JSRV is the aetiological agent of SPA. Although most type D retroviruses appear not to be pathogenic, a simian type D retrovirus (SRV 2) has been associated aetiologically with retroperitoneal fibromatosis in macaques (Bryant et al., 1986) and the other sheep and goat type D-related viruses have been implicated in epithelial tumours (De las Heras et al., 1991, 1993, 1995; Vitellozzi et al., 1993). Thus, in contrast to other retrovirus models involving leukaemogenic viruses, these sheep and goat retroviruses may represent unique models of oncogenic retroviruses associated with the development of naturally occurring epithelial carcinomas (Cremer & Gruber, 1992).

Whilst the circumstantial evidence implicating JSRV in neoplasia is compelling, the existing information does not rule out other possibilities in which it may, or may not, be involved in the neoplastic processes. JSRV may be acting only as a helper for some other replication-defective, acutely transforming retrovirus or result from reactivation of endogenous viral sequences as a consequence of neoplasia, as seen with other endogenous retroviruses (Weiss et al., 1985). The presence of up to 15 to 20 copies per genome of sequences related to the gag, pol and env genes of JSRV (York et al., 1992; Hecht et al., 1994) introduces major complications, particularly as endogenous retroviruses have been demonstrated in mice, chickens and cats to be involved in recombination events leading to the generation of oncogenic retroviruses (Stoye et al., 1991; Golovkina et al., 1994; McDougall et al., 1994; Bai et al., 1995). The occurrence of such events in SPA could be indicated by the apparent lack of serum antibodies to JSRV-CA in affected sheep or their unaffected flockmates (Sharp & Herring, 1983). It is clear, therefore, that further studies will be required to determine the role of JSRV in the aetiology of SPA and
the relationship between the exogenous and endogenous viruses.

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References


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