Jaagsiekte sheep retrovirus can be detected in the peripheral blood during the pre-clinical period of sheep pulmonary adenomatosis


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Peripheral blood leukocytes (PBLs) and tissue samples from 36 sheep were examined for jaagsiekte sheep retrovirus (JSRV) by hemi-nested PCR. Animals were classified according to the status of sheep pulmonary adenomatosis (SPA), which was confirmed by pathological examination, as follows: (i) sheep with classical SPA (cSPA, n = 10), (ii) sheep with atypical SPA (aSPA, n = 6), (iii) non-affected sheep from SPA-affected flocks (in-contact, n = 10) and (iv) non-affected sheep from SPA-free flocks (control, n = 10). JSRV proviral DNA was detected in the PBLs of 10/10 cSPA, 5/6 aSPA, 4/10 in-contact and 0/10 control sheep. Lung tumours and lymphoid organs were also found to be JSRV-positive. The number of positive PCR results was greater for sheep in the cSPA group than for those in the aSPA and in-contact groups. For the first time, it is concluded that JSRV can be detected in naturally infected sheep before the onset of clinical disease and even before the development of discernible tumours.

Sheep pulmonary adenomatosis (SPA, also known as jaagsiekte) is a worldwide transmissible lung cancer of sheep that was shown recently to be caused by an exogenous type D retrovirus, jaagsiekte sheep retrovirus (JSRV; Palmarini et al., 1999). To date, diagnosis has been possible only when clinical signs become apparent and not during the pre-clinical period of the disease. This is due to a lack of circulating JSRV-specific antibodies, as demonstrated by Western blotting (Sharp & Herring 1983; Ortín et al., 1998), and to the failure to detect JSRV proteins outside the lung tumour, as demonstrated by blocking ELISA and immunohistochemistry techniques (Palmarini et al., 1995).

Using RT–PCR, Palmarini et al. (1996) demonstrated the dissemination of JSRV in tissues of the lymphoreticular system (LRS) and in blood mononuclear cells of terminally ill sheep affected with both natural and experimental SPA. Improved PCR techniques have allowed the detection of JSRV proviral DNA in LRS tissues and have shown that viraemia can precede SPA tumour development in experimentally infected sheep (Holland et al., 1999; García-Goti et al., 2000). In this paper, we describe the detection of JSRV proviral DNA in the blood and LRS tissues of clinically affected and unaffected sheep with pathological evidence of natural SPA and in the same range of samples of SPA in-contact animals (non-affected sheep from SPA-affected flocks) with no evidence of pulmonary neoplasia.

A total of 36 Latxa ewes from commercial flocks was selected as part of a wider study into chronic respiratory diseases (CRD). These sheep were classified into four groups according to their flock of origin, clinical status and gross and histopathological pulmonary lesions. These latter features have been described in detail elsewhere (González, 1990; García-Goti et al., 2000). Sheep were grouped into: (i) classical SPA (cSPA; ten sheep with clinical evidence of CRD, all with large, diffuse and fluid-productive adenocarcinomas), (ii) atypical SPA (aSPA; four clinically normal sheep and two CRD-affected sheep with advanced lesions of maedi; all six had small solitary or multifocal, white, dry, circumscribed lung tumour nodules), (iii) in-contact (three clinically normal sheep and seven sheep affected by maedi; none of them had gross or histological evidence of neoplastic lung lesions), and (iv) controls (ten clinically normal sheep, with no gross or histological evidence of pulmonary lesions of either SPA or maedi).

The 26 sheep in the first three groups came from five different flocks with a history of SPA. The ten sheep in the control group came from two flocks in which no clinical or...
pathological evidence of SPA had been found in the preceding 16 years. The mean ages of the sheep in each of the groups were 5-6, 5-3, 6-9 and 8-0 years, respectively.

Before necropsy, peripheral blood was collected into EDTA tubes and peripheral blood leukocytes (PBLs) were isolated by centrifugation after lysis of erythrocytes (García-Goti, 1999); the resulting cell pellets were stored at -80 °C. Sheep were then humanely culled and examined macroscopically. Tissues for PCR examination were taken with separate sterile instruments in the following order: brain, kidney, mammary gland, mammary lymph node, spleen, mediastinal lymph node, non-neoplastic areas of the lung and, finally, neoplastic areas (if present). Tissue samples were placed into individual vials, immediately frozen in liquid nitrogen and stored at -80 °C. Samples of the adjacent areas of the same tissues were also taken and placed in 10% buffered formalin for subsequent histopathological examination, according to routine procedures.

A hemi-nested PCR that was specific for the U3 region of exogenous JSRV (U3-hn-PCR) was used to detect JSRV proviral DNA in the blood and tissue samples, as described previously (Palmarini et al., 1996; García-Goti et al., 2000). Briefly, tissue samples and PBL pellets were thawed and homogenized. Homogenates were then resuspended in EDTA and Tris–HCl (pH 8.0), incubated with proteinase K and boiled. Total DNA was extracted by a modified phenol–chloroform method (Sambrook et al., 1989). Primers PI and PIII were used in the first round of PCR amplification and primers PI and PVI were used in the second round to amplify a 133 bp sequence. PCR products were visualized following electrophoresis in agarose gels stained with ethidium bromide.

U3-hn-PCR detects a single copy of JSRV provirus in a background of 500 ng of normal sheep DNA (Palmarini et al., 1996). In the present study, six × 300 ng replicates of each DNA sample (a total of 1.8 µg) were tested in order to increase the sensitivity of the PCR procedure and to quantify the results.

The number of positive PCR replicates obtained from the six tested was compared using the InStat statistics package (GraphPad Software). Due to constraints in the SD in the different groups (Bartlett test) and in the normality of the data (Kolmogorov–Smirnov test), a non-parametric analysis of variance (Kruskal–Wallis test and Dunn’s Multiple Comparisons test) was used to compare the median PCR values of the different groups. Two comparisons were performed, one between groups of sheep (as defined above) for the same type of sample and the other between the different tissue samples within each group of sheep. PCR results of kidney, brain and mammary gland were all grouped as extra-thoracic non-lymphoid (ENL) organs.

The results of JSRV proviral DNA detection in the blood and tissue samples of sheep from the four different groups are detailed in Table 1. Overall, JSRV was detected in all 16 SPA sheep (classical and atypical) and in 8/10 SPA in-contact sheep. None of the 71 samples tested from the 10 control sheep was positive following U3-hn-PCR. JSRV was detected in PBLs, lung tumour, mediastinal lymph node and non-neoplastic lung from all cSPA sheep and in a lower proportion of the spleen and mammary lymph node samples. Proviral DNA was detected in all lung tumour samples from aSPA sheep, in all but one of the PBL samples, mediastinal lymph nodes and non-neoplastic lungs, and in a lower proportion of spleen and

<table>
<thead>
<tr>
<th>Sample</th>
<th>Classical</th>
<th>Atypical</th>
<th>In-contact</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBLs</td>
<td>10/10 (100)</td>
<td>5/6 (83)</td>
<td>4/10 (40)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>0/10 (0)</td>
<td>0/6 (0)</td>
<td>0/5 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Brain</td>
<td>0/9 (0)</td>
<td>0/6 (0)</td>
<td>0/5 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Kidney</td>
<td>2/10 (20)</td>
<td>0/6 (0)</td>
<td>0/1 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Spleen</td>
<td>4/10 (40)</td>
<td>2/6 (33)</td>
<td>2/10 (20)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Mammary lymph node</td>
<td>5/10 (50)</td>
<td>1/6 (17)</td>
<td>1/9 (11)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Non-neoplastic lung</td>
<td>10/10 (100)</td>
<td>3/4 (75)</td>
<td>1/10 (10)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Mediastinal lymph node</td>
<td>10/10 (100)</td>
<td>5/6 (83)</td>
<td>2/10 (20)</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Lung tumour</td>
<td>10/10 (100)</td>
<td>6/6 (100)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Total sheep</td>
<td>10/10 (100)</td>
<td>6/6 (100)</td>
<td>8/10 (80)</td>
<td>0/10 (0)</td>
</tr>
</tbody>
</table>

NA, Not available.

Table 1. Distribution of JSRV in the blood and tissue samples of sheep classified according to their SPA status

Results are expressed as the number of JSRV-positive samples or sheep/number tested. Percentages are shown in parentheses.
mammary lymph node samples. JSRV was detected in 40% of PBLs of SPA in-contact sheep and in a lower proportion of the mediastinal lymph node, spleen, mammary lymph node and non-neoplastic lung samples.

Differences among the four groups of sheep were also observed with regard to the number of positive PCR replicates obtained from the different samples tested (Fig. 1). The control group of sheep was not considered in statistical analyses as no positive PCR results were obtained from any sample tested. Differences between the other three groups (cSPA, aSPA and in-contact) were not found with respect to the ENL organs (mammary gland, brain and kidney), which were completely negative with the exception of two cSPA sheep. In contrast, lung tumour samples were always positive from either cSPA or aSPA sheep. Considering the remaining five samples together (non-neoplastic lung, mediastinal lymph node, mammary lymph node, spleen and PBLs), samples from the cSPA sheep had larger numbers of positive PCR replicates (151/300, 50.3%) than the aSPA sheep (42/168, 25.0%) and the eight positive in-contact sheep (16/234, 6.8%). Differences, however, were only significant (P < 0.001) between the cSPA and the in-contact sheep. Considering the samples individually, the number of positive replicates obtained from mediastinal lymph node, non-neoplastic lung and PBLs was significantly larger (P < 0.001) in the cSPA group (95.0, 73.3 and 58.3%, respectively) than in the in-contact group (6.2, 4.2 and 14.6%, respectively). The same samples from the aSPA group gave an intermediate number of positive PCR results (52.8, 29.2 and 25.0%), which did not differ significantly from those of the other two groups. The number of positive PCR replicates obtained from spleen and mammary lymph node did not differ significantly between any of the three groups of sheep.

Differences in the number of positive replicates also were found when comparing different samples within each group. Within the JSRV-positive sheep of the in-contact group, PBLs yielded the largest number of positive replicates (1416%), but these were not significantly different from those of the other samples. Within the aSPA sheep, lung tumour samples gave a significantly larger (P < 0.001) number of positive PCR replicates (100%) than mammary lymph node (13-9%), spleen (5-6%) and ENL organs (0%). The mediastinal lymph node, non-neoplastic lung and PBLs in this group showed an intermediate situation and were not significantly different from the other samples. In the cSPA group, lung tumour, mediastinal lymph node and non-neoplastic lung samples also yielded a significantly larger (P < 0.001) number of positive PCR replicates (100, 95.0 and 73.3%, respectively) than mammary lymph node, spleen and ENL samples (15, 10 and 2.2%, respectively). Results from the PBL samples in this group were intermediate and numbers were only significantly larger (P < 0.001) than those from the ENL organs (Fig. 1).

Previous studies have demonstrated the presence of JSRV in the blood of sheep with experimentally induced and natural SPA (Palmarini et al., 1996; Holland et al., 1999). The present report clearly shows that JSRV can be detected in naturally infected live sheep before the onset of clinical SPA and even in the absence of discernible lung tumours. Although only a small number of in-contact sheep were examined, it seems clear that subclinical JSRV infection can reach a high prevalence in flocks affected with SPA.

In agreement with previous reports (Palmarini et al., 1996; Holland et al., 1999), dissemination of JSRV infection outside the lung appears to be restricted to LRS organs and PBLs, whereas non-lymphoid organs remain mostly negative. Within the LRS, the mediastinal lymph node presents the
highest proportion of JSRV infection in animals with lung tumours, but not in in-contact sheep. This may be due to the role of this lymph node in the local drainage from the lung and to the presence of tumour metastases (García-Goti et al., 2000). The detection of JSRV in the blood also deserves attention, as this sample shows the largest number of positive results among those samples not related to the respiratory tract and, in fact, the largest overall in the in-contact group of sheep. Further studies are needed to clarify whether LRS infection is a necessary phase in SPA, similar to that for mouse mammary tumours of retroviral aetiology (Callahan & Smith, 2000), and whether it compromises the host immune response.

Overall, the neoplastic lung is the tissue that gives the largest proportion of positive PCR results, both in terms of sheep and replicates, a finding that is in agreement with previous reports on JSRV detection by immunohistochemistry or blocking ELISA techniques (Palmarini et al., 1995). Our results also indicate that LRS dissemination of JSRV is pronounced in animals with large, fluid-producing tumours (cSPA), intermediate in sheep with small, circumscribed, dry tumour nodules (aSPA) and low in in-contact unaffected sheep. Statistical analyses were not, in all cases, conclusive in this respect, but this might be a consequence of the use of non-parametric tests, which, although the most appropriate for our data, may lack the power to identify real differences between groups. Bearing this in mind, we hypothesize that JSRV dissemination is very low during the pre-clinical stages of infection and increases following the initiation and development of neoplastic lesions due to active virus replication in the tumour cells. However, the precise factors that regulate the genesis and progression of the tumour remain to be elucidated.

With regard to the detection of JSRV proviral DNA in the blood of clinically affected sheep, the performance of the PCR protocol used in this study (10/10 positive sheep with 2/6 to 6/6 positive replicates) is better than that previously reported by Palmarini et al. (1996) (0/6 sheep) and Holland et al. (1999) (5/7 sheep with 1/8 to 2/8 positive replicates). However, the potential for its use in the detection of subclinical infection in live animals seems to be compromised by the low levels of infected blood cells at this stage. In our study, JSRV was detected in the PBLs of only 4/8 positive in-contact sheep and three of them gave only one positive PCR replicate. These figures are in agreement with the estimated low frequency of JSRV infection of blood mononuclear cells in clinically affected sheep (less than 1/240,000; Holland et al., 1999) and with the fluctuating positive PCR results obtained in a prospective field study on JSRV viraemia in clinically normal sheep from an SPA-affected flock (García-Goti, 1999). Nevertheless, the development of a more sensitive, high-throughput PCR test that would allow repeated testing would greatly facilitate the realization of longitudinal surveys in affected flocks and permit the identification of routes and risk factors of transmission of JSRV infection.

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