

Jaagsiekte Retrovirus Is Widely Distributed both in T and B Lymphocytes and in Mononuclear Phagocytes of Sheep with Naturally and Experimentally Acquired Pulmonary Adenomatosis

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Jaagsiekte sheep retrovirus (JSRV) is a type D retrovirus specifically associated with a contagious lung tumor of sheep, sheep pulmonary adenomatosis (SPA). JSRV replicates actively in the transformed epithelial cells of the lung, and JSRV DNA and RNA have been detected in lymphoid tissues of naturally affected animals. To determine the lymphoid target cells of JSRV, CD4⁺ T cells, CD8⁺ T cells, B lymphocytes, and adherent cell (macrophage/monocyte) populations were isolated from the mediastinal lymph nodes of naturally affected sheep and lambs inoculated with JSRV. Cells were enriched to high purity and then analyzed for JSRV proviral DNA by heminested PCR, and the proviral burden was quantitated by limiting dilution analysis. JSRV proviral DNA was found in all subsets examined but not in appropriate negative controls. In sheep naturally affected with SPA, JSRV proviral burden was greatest in the adherent cell population. In the nonadherent lymphocyte population, surface immunoglobulin-positive B cells contained the greatest proviral burden, while CD4⁺ and CD8⁺ T cells contained the lowest levels of JSRV proviral DNA. In most of the cases (5 of 8), provirus also could be detected in the peripheral blood mononuclear cell (PBMC) population. A kinetic study of JSRV infection in the mediastinal lymphocyte population of newborn lambs inoculated with JSRV found that JSRV proviral DNA could be detected as early as 7 days postinoculation before the onset of pulmonary adenomatosis, although the proviral burden was greatly reduced compared to adult natural cases. This was reflected in the levels found in PBMC since proviral DNA was detected in 2 of 13 animals. At the early time points studied (7 to 28 days postinoculation) no one subset was preferentially infected. These data indicate that JSRV can infect lymphoid and phagocytic mononuclear cells of sheep and that dissemination precedes tumor formation. Infection of lymphoid tissue, therefore, may play an important role in the pathogenesis of SPA.

Jaagsiekte sheep retrovirus (JSRV) is an exogenous type D retrovirus specifically associated with a contagious lung tumor of sheep, known as sheep pulmonary adenomatosis (SPA). SPA represents a unique model of lung neoplasia and studies on its etiopathogenesis can yield further insights into the causes and mechanisms of lung and epithelial neoplasms (7, 24, 29). JSRV is distinct from the transcriptionally active endogenous retroviruses present in the ovine genome and has been detected only in sheep affected by SPA (1, 8, 22, 25). The main sites of viral replication and assembly are transformed epithelial cells of the lungs (23). In addition, low levels of viral RNA and DNA have been detected by a JSRV-specific PCR in

several lymphoid tissues of affected sheep (25). Many aspects in the pathogenesis and oncogenesis of SPA require clarification. Among these, the sites of JSRV replication and the interaction between the virus and the host immune system require additional investigation.

Natural infection with JSRV is characterized in SPA-affected animals by the immunologically silent nature of infection, highlighted by an apparent absence of a specific humoral response (21, 31, 34). However, this remains a controversial issue since some studies claim there is evidence to indicate local immunoglobulin A responses, formation of viral immune complexes, and systemic antibody responses that are cross-reactive with recombinant antigens of highly related viruses (18, 28, 35, 36). Thus far, JSRV-specific cellular immune responses have not been examined. Further evidence that JSRV infection may have an intimate involvement with lymphoid tissue and the ensuing immune response is suggested by both a local and a peripheral reduction in CD4⁺ T lymphocytes, cells central to the regulation of immune responses (27). As yet, the lymphoid target cells of JSRV infection are unknown and, consequently, the cell types that JSRV utilizes in the dispersal, dissemination, and progression of SPA also are unknown.

The aims of this study were to identify the lymphoid cells infected by JSRV in vivo, to estimate the proviral load in lymphoid

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TABLE 1. Mean purities of isolated cells from mediastinal lymph nodes after mini-MACS separation

Antibody clone	Specificity	% Mean purity (range) of cells from animals:	
		With naturally acquired SPA ^a	Inoculated with JSRV ^b
17D	CD4	97.0 (92.6–99.0)	91.2 (79.0–98.2)
7C2	CD8	94.8 (87.0–98.0)	74.4 (58.1–99.8)
VPM30	B	95.4 (95.0–98.2)	78.3 (53.6–97.6)

^a Cells were isolated from animals with naturally acquired SPA.

^b Cells were isolated from newborn lambs inoculated with JSRV.

phoid subsets, and to establish whether lymphoid infection precedes or is consequent to alveolar transformation.

MATERIALS AND METHODS

Animals and experimental design. Studies were conducted with eight sheep naturally affected by SPA in which the clinical diagnosis of SPA was confirmed by macroscopic and histological examination of the lungs at necropsy. In addition, 13 colostrum-fed, newborn lambs were infected with concentrated lung fluid collected from SPA-affected sheep (30). The inoculum was prepared from a single batch of lung fluid that was divided into aliquots and stored at -70°C until the lambs were inoculated. This ensured that each lamb received the same dose of JSRV. Lambs were euthanized by an intravenous overdose of pentobarbitone before the onset of clinical signs at 7, 14, and 21 days postinoculation (d.p.i.) (three lambs per time point) and at 28 d.p.i. (four lambs). Macroscopic and histological examination of the lungs at necropsy was performed to identify any gross histopathological changes. Mediastinal lymph nodes were removed, and portions were fixed in formalin for the immunohistochemical studies. The remainder of the node was placed in sterile medium until processed, within 2 h, to obtain lymphocytes for analysis.

Isolation of lymphocytes. Mediastinal lymph nodes were dispersed by gentle disruption through a large-gauge metal sieve to form a single cell suspension. Overnight plastic adherence was used to enrich the cell population for macrophages. Nonadherent cells were removed by washing with complete tissue culture growth medium. Monoclonal antibodies 17D, 7C2 (supplied by W. Hein, Basel Institute, Basel, Switzerland), and VPM30 (supplied by J. Hopkins, Veterinary Pathology Department, University of Edinburgh) (11, 17, 20) were used with mini-MACS (Miltenyi Biotec, Surrey, United Kingdom) to isolate CD4⁺ T cells, CD8⁺ T cells, and surface immunoglobulin-positive B cells, respectively. Antibodies were used at the optimal dilution as determined by immunofluorescence staining. Fluorescence-activated cell sorting analysis with the active gate set on the total cell population was performed on the mini-MACS-separated cells to verify the efficacy of the separation procedure, and the data were analyzed by using Lysis II software (Becton Dickinson, Oxford, United Kingdom). Peripheral blood mononuclear cells (PBMC) from sheep with naturally acquired SPA as well as from the inoculated newborn lambs were isolated by centrifugation over Lymphoprep (Nycomed, Birmingham, United Kingdom) (25), and the cells were labelled with the appropriate antibodies. The purity of the separated cells is given in Table 1.

Total leukocyte counts were made by using the standard electronic Coulter Counter procedure (Coulter Electronics Ltd., Herts, United Kingdom). Differential counts were estimated by light microscopy after May-Grünwald Giemsa staining of methanol-fixed whole-blood thin films (12).

Source and preparation of DNA. Genomic DNA was extracted from the purified subsets (1×10^6 to 10×10^6 cells) and a jaagsiekte tumor cell line, Js7 (14), by using the Qiagen Tissue Amp kit (Qiagen Ltd., Surrey, United Kingdom), and plasmid DNA was purified by using Qiagen spin columns according to the manufacturer's specifications.

LDA. The frequency of JSRV proviral sequences was estimated by limiting-dilution analysis (LDA) by using a heminested PCR (hn-PCR) that amplified a portion of the U3 long terminal repeat (LTR) (25). The LDA assay was validated with template DNA prepared from the Js7 cell line and plasmid pLTR-gag containing the upstream LTR of JSRV. A minimum of eight twofold dilutions of test DNA, starting at 200 ng per PCR, were made in $1 \times$ PCR buffer containing a constant amount of bacteriophage λ DNA to maintain equivalence in each PCR. Eight replicates for each dilution were amplified by hn-PCR, and the size of the product was determined by electrophoresis through 2% agarose gels. Each reaction was scored as positive or negative, and the proviral load was estimated by using the null class of the Poisson distribution. The most probable weight (MPW) of DNA containing a single provirus was calculated as indicated by statistical methods. Three to five negative controls of either water or DNA isolated from unaffected normal sheep were included in each test to rule out possible cross-contamination during the DNA extraction and amplification procedures.

Statistical methods. Data were analyzed with a Bayesian Monte-Carlo Markov Chain-based statistical analysis package, WinBugs, developed by researchers at the United Kingdom Medical Research Council Biostatistics Unit (4). (WinBugs may be downloaded from website <http://www.mrc-bsu.cam.ac.uk/bugs/Wel-come.html>.) A standard complementary log-log model was used within WinBugs to estimate the MPW and the 95% confidence intervals of DNA which would contain a single provirus. (A copy of the WinBugs model used is available at <ftp://ftp.bioss.sari.ac.uk/ia/in/assay.odc>.) The results obtained were found to be unaffected by the prior choice of the Bayesian method and were broadly comparable to those produced by the DILUTION procedure within the Genstat statistical package (26).

The MPW of DNA containing a single provirus in different types of cells isolated from different animals were compared by using Genstat (26). Assays for which the model had a poor fit were excluded from all further analyses. Statistical comparison of the mean logarithm of the MPW from each cell type was done by using the Residual Maximum Likelihood directives within Genstat weighted by the inverse square of the standard error. This ensured that imprecise estimates were given less weight in the analysis. Animals were treated as random effects, while the cell type was treated as a fixed effect. The multiple comparisons between the different cell types were controlled by using the Bonferroni inequality.

IHC. Tissues were fixed in 10% neutral buffered formalin, processed routinely with an automatic tissue processor, and embedded in paraffin. Four- to 6- μm tissue sections from each sample were examined for JSRV-CA by the immunohistochemistry (IHC) protocol reported previously (23), except that an antigen retrieval step was also included. This additional procedure consisted of heating the sections by microwave at 800 W two times for 7 min. The SPA tumor was used as a positive control, and lymph nodes collected from unaffected controls were used as negative controls.

RESULTS

Distribution of JSRV in lymphoid cells of SPA-affected animals: natural disease. To establish the sensitivity and reproducibility of the LDA assay, preliminary experiments were performed with a known number of copies of pLTR-gag and Js7 genomic DNA. Under optimized conditions and with pLTR-gag as a target, LDA estimated that the amount of DNA equivalent to a single copy of plasmid was 6.553×10^{-18} g of input DNA, which is equivalent to 1.2 copies of the 5,067-bp plasmid. In three independent LDAs performed with Js7 DNA, the estimated amount of DNA containing one copy of the target sequence (i.e., a single copy of JSRV-U3) was 5.98 ± 0.45 pg. If we assume that the weight of the genomic DNA contained in one cell is 6.66 pg, the estimated proviral frequency in Js7 cells was 0.90 ± 0.07 copies/cell. These preliminary experiments confirmed that the hn-PCR was sufficiently sensitive to detect a single copy of JSRV and was highly reproducible.

The analysis of the separated lymphoid cells isolated from eight sheep with naturally acquired SPA revealed that JSRV was widely distributed. JSRV was detected in each of the four subsets tested (Table 2). With the exception of one animal, the JSRV proviral load was higher in adherent cells than in non-

TABLE 2. Number of copies of proviral JSRV in lymphoid cells from sheep with naturally acquired SPA

Animal no.	No. of copies (subset purity [%]) with cell phenotype:			
	CD4 ⁺	CD8 ⁺	B cells (sIg) ^c	Adherent cells
JA065	NP ^a (98.8)	ND ^b	ND	0.53
JA087	5.92 (96.3)	ND	ND	0.69
JA089	0.37 (99)	0.38 (97.5)	ND	6.32
JA090	0.35 (98.1)	0.92 (95.4)	ND	4.15
JA101	0.95 (97.6)	0.70 (95.1)	1.53 (95.6)	7.46
JA103	0.10 (98)	0.29 (87)	0.92 (98.2)	1.02
JA104	9.91 (92.6)	3.05 (96)	12.9 (95.6)	26.3
JA114	NP (95.8)	0.06 (98)	0.69 (95)	1.17

^a NP, no positive hn-PCRs occurred at any level of input DNA (maximum input DNA = 125 ng \times eight replicates).

^b ND, not done (subset not isolated).

^c sIg, surface immunoglobulin.

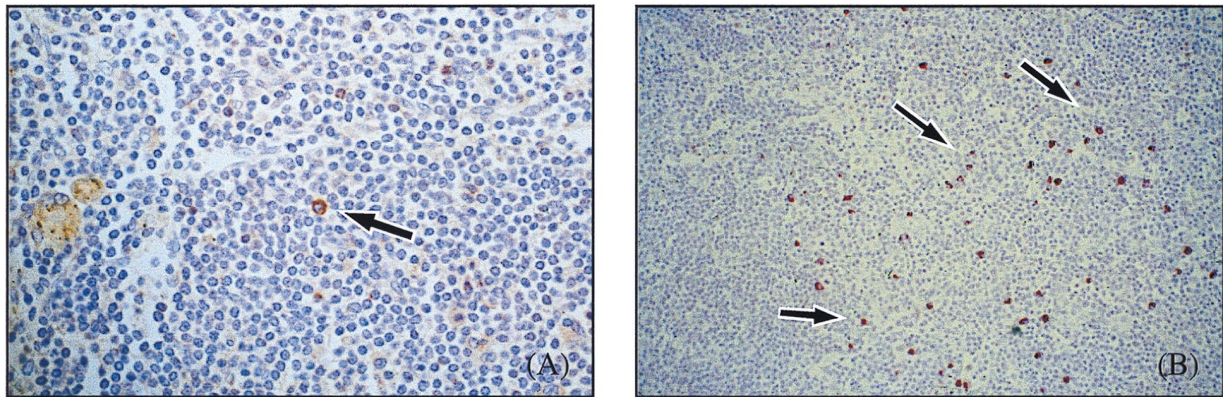


FIG. 1. Immunohistochemistry of a tracheobronchial lymph node of sheep naturally affected by SPA. The dark staining represents the positive reaction. (A) A typical result is shown, with only one positive mononuclear cell present in the field (magnification, $\times 400$). (B) Several positive cells in the paracortical area of the lymph node (magnification, $\times 100$) are shown.

adherent lymphocytes. The average proviral load in adherent cells was 5.95/100 ng of DNA, which is approximately equal to 1 copy of JSRV provirus per 2,524 cells based on the estimation that 100 ng of DNA is approximately equivalent to 15,000 cells. Within the lymphocyte population, the proviral load was greatest in surface immunoglobulin-positive B cells (1/3,768 cells) compared to CD4⁺ cells (1/6,825 cells) and CD8⁺ cells (1/16,683 cells). Statistically significant differences were present between at least some of the groups ($P < 0.001$). Differences were identified by using the Bonferroni correction between adherent cells and CD8 cells ($P = 0.012$), and differences close to statistical significance were observed between adherent cells and CD4 cells ($P = 0.09$). No other comparisons were found to be near significance.

Since JSRV proviral DNA was detected in the mediastinal lymph node cells of all SPA cases, PBMC were analyzed for the presence of proviral DNA. By subjecting DNA isolated from PBMC to eight identical replicate reactions and thus testing a total of 1.6 μ g of DNA, positive reactions were identified in five of eight animals. The frequency of positive reactions in each affected animal was low (a maximum of two of eight replicates were positive), indicating a low proviral burden or frequency of infected cells ($< 1/240,000$ PBMC). The number of positive PCRs from PBMC samples did not correlate with the proviral burden in the mediastinal lymph node samples.

IHC. The IHC analysis was performed on thoracic lymph nodes from four sheep with natural SPA to verify whether JSRV proteins could be detected in these tissues. JSRV-CA was detected in the mediastinal and tracheobronchial lymph nodes of three animals. Usually only an isolated or very small number of positive mononuclear cells and a small proportion of positive-staining plasma cells were present in each section (Fig. 1A). Positive cells were found mainly in the paracortical zones and, in some cases, in the medullary sinuses. Occasionally, several positive cells could be found in a single section (Fig. 1B).

Immunophenotypic profiles of SPA-affected animals. To investigate possible phenotypic anomalies arising from infection, lymphoid cells isolated from peripheral blood and mediastinal lymph nodes were stained with the same monoclonal antibodies used for subset purification. In eight naturally SPA-affected animals, the main characteristic was a mean reduction in the levels of CD4⁺ T lymphocytes in PBMC (absolute number per milliliter for CD4⁺ cells: mean = 4.7 [range, 1.98 to 12.9] $\times 10^5$ /ml) in the majority of cases compared to the normal values from unaffected sheep (CD4⁺ cells: 9.1 [5.2 to 10.4] $\times 10^5$ /ml).

An overall increase in the total number of circulating leukocytes in SPA-affected animals (mean = 14.8 [range, 12 to 17.1] $\times 10^6$ /ml) was also observed compared to levels in unaffected sheep (8 [4 to 12] $\times 10^6$ /ml). Immunostaining of isolated mediastinal lymph node cells revealed no gross anomalies in the frequency of the cell types examined, although CD4⁺ and CD8⁺ T-cell levels (%CD4⁺ cells = 55 [48 to 64]; %CD8⁺ cells = 21 [18 to 26]) were slightly elevated compared with normal levels for lymph nodes of unaffected animals (%CD4⁺ cells = 43 [22 to 49]; %CD8⁺ cells = 14 [12 to 20]). The proviral burden in each subset did not correlate with the anomalies in PBMC frequency, suggesting that subsets with the greatest proviral burden were not expanded or depleted.

JSRV proviral load in experimentally infected lambs. Low levels of provirus were detected in the majority of the samples. For reasons which are unclear, lower mean purities of CD8⁺ and sheep immunoglobulin-positive B cells were obtained by mini-MACS separation in some samples from neonatal lambs. However, in some cases high-purity separations were achieved, the differences possibly related to the maturity of the lymphoid cells. At the lowest dilution of DNA, a range of positive hn-PCRs was found (1 of 8 to 5 of 8), but this was not sufficient to allow quantitative PCR analysis of these samples. JSRV was detected in the lymphoid cells of each of the 13 inoculated animals between 7 and 28 d.p.i. (Table 3). Histopathological evidence of SPA was detected in two lambs at 21 and 28 d.p.i.,

TABLE 3. Detection of JSRV proviral DNA in cells from mediastinal lymph nodes and PBMC of experimentally infected lambs

Cell type	No. of replicates positive for JSRV at:												
	7 d.p.i. in animal no.:			14 d.p.i. in animal no.:			21 d.p.i. in animal no.:			28 d.p.i. in animal no.:			
	1	2	3	4	5	6	7	8 ^a	9	10	11	12	13 ^a
CD4 ⁺	1	0	0	0	0	2	0	0	0	1	2	3	2
CD8 ⁺	0	0	1	2	0	2	1	2	0	1	1	1	0
B cells	0	0	1	0	0	2	1	1	1	0	1	0	1
Adherent cells	0	1	2	0	1	5	0	0	3	1	1	1	5
PBMC	0	0	0	0	0	1	0	0	0	2	0	0	0

^a Lambs with histological evidence of SPA. Each reaction contained 100 ng of genomic DNA and eight replicates per sample.

indicating a rapid onset of disease and demonstrating that systemic lymphatic dissemination could occur before tumor formation.

DISCUSSION

JSRV has been detected exclusively in sheep affected by SPA and, although it replicates principally in the transformed epithelial cells of the lungs, widespread infection of the lymphoreticular tissues is established. The interplay between JSRV and the immune system of the host is not clear. In previous studies on naturally and experimentally JSRV-infected sheep, viral transcripts and proviral DNA were detected in mediastinal and peripheral lymph nodes, spleen, bone marrow, and thymus (25). Identification of immune-system cells infected by JSRV *in vivo* can reveal important aspects of the pathogenesis of SPA.

JSRV most likely establishes an infection of lymphoid cells and mononuclear phagocytes of SPA-affected sheep and, in general, the proviral load is higher in adherent cells than in B cells and CD4⁺ or CD8⁺ T cells. Although a rare event in the majority of the thoracic lymph nodes studied, IHC results clearly identify lymphoid cells that express JSRV viral antigen. While *in situ* hybridization with virus-specific probes and immunophenotypic labelling could provide definitive evidence of the cell types infected, the level of infection is not easily quantifiable by this method.

An interesting feature of SPA-affected animals is the increased numbers of macrophages in the lungs (13, 15, 36), and it is this cell type which contains the greatest proviral burden. Since we did not examine isolated subsets for viral RNA, it is possible that the levels observed in macrophages represent phagocytized cellular debris containing JSRV proviral DNA. However, previous studies focusing on JSRV expression in the lymphatic tissue (24) and the IHC studies described here point to infection and viral expression in nontumor-nonlineg tissues. Furthermore, unidentified JSRV hn-PCR-positive cells which copurify with each of the subsets cannot account for the differences in the estimates of the proviral burden in naturally affected sheep unless each contaminating cell contains multiple copies of proviral DNA. Cells isolated from animal JA101 contain 7.46 proviral copies/100 ng of DNA in the least-pure adherent-cell population. If we assume that this is the maximum proviral burden, contamination of each purified subset would predict values significantly smaller than the actual values obtained. This finding is consistent within individual animals and with the group as a whole. Contamination by unidentified infected cells cannot therefore account for the observed differences.

In experimentally infected lambs, JSRV proviral DNA can be detected in lymphoid cells as early as 7 d.p.i. when no histological signs of SPA are present. This indicates that infection of lymphoid cells precedes the neoplastic transformation. It is not clear if infection of lymphoid and/or resident macrophages in the lungs precedes or follows the infection of the cells from which the neoplasm originates. Preliminary findings in naturally infected flocks indicate that proviral DNA can be detected before the onset of clinical signs (3a).

The infection of the immune system by JSRV may be advantageous to the virus either by directly facilitating the infection and subsequent transformation of epithelial cells or indirectly by the induction of an immunosuppressive state in the host. The first scenario can be deduced by the notable similarities between JSRV infection in sheep and mouse mammary tumor virus (MMTV) infection in mice. Both MMTV and JSRV are exogenous viruses with endogenous counterparts

and are associated with epithelial tumors, an uncommon feature among retrovirus-induced neoplasias. Each virus replicates actively in transformed epithelial cells but also maintains a low-level infection of host lymphoid cells. In the MMTV model, the infection and interaction of B and T cells, which is mediated by the viral superantigen, is an absolute requirement for the virus to reach the mammary gland and induce transformation (5, 9, 10, 16, 33). Indeed, both B and T cells of MMTV-infected mice shed infectious viral particles (3). The pathogenesis of MMTV-induced mammary carcinoma and SPA differs in an important aspect: the sites of entry of the two viruses with respect to the accessibility of their target cells for transformation. While MMTV enters through the digestive tract and then has to "travel" to reach the mammary gland, JSRV most probably enters through the respiratory route, and therefore type II pneumocytes and the Clara cells are readily accessible. Although initial infection of the upper respiratory tract (e.g., the tonsils) cannot be ruled out, it is conceivable that JSRV first infects the epithelial cells of the lungs (since intratracheal inoculation of lung fluid from SPA-affected sheep results in experimental reproduction of SPA), where it is able to actively replicate. After a few rounds of replication, infected lymphoid or phagocytic cells may carry JSRV to the mediastinal lymph nodes. At this point JSRV-infected cells could be a depot for the infection of other lymphocytes and then, through the recirculation of these cells, infection would be disseminated to other lymphoid tissues.

The infection of lymphoid cells and mononuclear phagocytes by JSRV may impair the normal function of these cells and affect the host immune response. However, as yet this is not formally demonstrated, but it may be an effective mechanism by which to avoid immune responses. Other cell types which are vital in the generation of immune responses are dendritic cells (DC) and follicular dendritic cells. DC were not isolated for JSRV provirus detection. The infection of DC could be a mechanism exploited by JSRV to avoid an effective immune response (6), and this possibility requires investigation. DC or cells of another unidentified phenotype may represent a minor contaminating population of JSRV hn-PCR-positive cells within the mini-MACS-purified subsets. While it is not possible to completely exclude this possibility, it is unlikely that other cells of an unidentified phenotype could be responsible for the differences in viral load detected, since within an individual we would expect the levels of JSRV contaminating cells to be uniform across the subsets which were isolated. These experiments clearly identify lymphocyte subsets in which the purity is >98% and contain the greatest proviral load.

The majority of cases of naturally acquired SPA had a peripheral CD4⁺ lymphocytopenia and neutrophilia compared to levels estimated from a large sample size of healthy sheep (6a, 19), confirming a previous report conducted on a small number of SPA cases (27). SPA-affected sheep lack circulating anti-JSRV antibodies and have an increased susceptibility to secondary bacterial infection (32, 34). These observations could indicate that SPA-affected sheep are immunocompromised. Furthermore, reduced mitogen responses of PBMC isolated from animals with SPA compared to age-matched unaffected controls were found (unpublished observations). This is consistent with other retroviruses which are responsible, by different pathogenic mechanisms, for the direct induction of immune disorders in diverse animal species (2).

In the SPA model it is difficult to attribute the apparent lack of JSRV antiviral responses directly to JSRV. These observations were made from samples isolated from sheep with obvious clinical signs of SPA. At this stage, the extensive lesions in

the lungs, the frequent secondary bacterial infections, and the consequent respiratory impairment could be, by themselves, responsible for the absence of detectable responses. More studies are needed to dissect the immune response in SPA-infected sheep and to specifically evaluate the cellular immune response to JSRV. Propagation of JSRV *in vitro* and the development of an infectious molecular clone would greatly facilitate work in this area.

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