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Lentivirus-Induced Lymphoproliferative Disease

Comparative Pathogenicity of Phenotypically Distinct Ovine Lentivirus Strains

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For investigation of the pathogenicity of lentivirus strains, which have distinctly different cytopathic phenotypes in synovial membrane cell culture, plaque-purified, lytic, and nonlytic ovine lentivirus (OvLV) isolates were inoculated intratracheally into two groups of neonatal lambs. Twelve lambs were inoculated with a lytic OvLV isolate and 3 lambs each with two nonlytic OvLV isolates. Five control lambs were inoculated with either virus-free medium or were left uninoculated. In 8 of 12 lambs inoculated with a lytic OvLV isolate mild to severe lesions of lymphoid interstitial pneumonia (LIP) and pulmonary lymphoid hyperplasia developed, 6 of 12 lambs had lesions of pulmonary lymph node follicular hyperplasia, 3 of 9 female lambs had lesions of lymphoproliferative mastitis, 3 of 10 lambs had lesions of lymphocytic/plasmacytic synovitis, and 3 lambs had no lesions. In 3 of 6 lambs inoculated with nonlytic OvLV isolates only mild LIP lesions developed, without concurrent mammary gland or joint lesions. Bronchoalveolar lavage samples from OvLV-diseased lambs contained on average 1.5-fold more numbers of total leukocytes, and 4-fold more numbers of lymphocytes, compared with bronchoalveolar lavage samples of normal lambs. Monoclonal antibodies to ovine lymphocyte surface markers showed that the SBU-T8$^+$ lymphocyte (CD 8 equivalent) was the predominant lymphocyte subset (mean of 65% of total lavaged lymphocytes) in bronchoalveolar lavage samples of 3 diseased lambs. Ovine lentivirus was reisolated from multiple tissues of both groups of OvLV-inoculated lambs, but the percentage of individual tissues infected was greater in lambs inoculated with the lytic viral isolate. Control lambs had no lesions and failed to produce OvLV-specific antibodies or yield OvLV from tissues. All OvLV-inoculated lambs produced either low or undetectable serum virus neutralizing antibodies. In contrast, lambs inoculated with either lytic or nonlytic OvLV produced precipitating antibodies to OvLV glycoprotein and group-specific protein. However, initial detection of precipitating antibodies to OvLV glycoprotein was earlier (mean, 5.8 weeks after inoculation) in OvLV-infected lambs in which severe lymphoproliferative disease developed and delayed (mean, 10.2 weeks after inoculation) in OvLV-infected lambs with mild or no lesions. Together, these results suggest that lentivirus isolates produced disease in a virus strain-dependent manner and suggest that humoral immune responses against OvLV failed to prevent lesion development in lentivirus-infected lambs. Pulmonary inoculation of neonatal lambs with a plaque-purified OvLV strain induced lymphoproliferative disease of the lung, mammary gland, and joints, suggesting that pathogenic lentivirus strains have multiple tissue tropisms and do not produce disease in a strictly organ-specific manner. The spectrum of pulmonary-associated lesions in lentivirus-inoculated lambs (lymphoid interstitial pneumonia, pulmonary lymphoid hyperplasia, and pulmonary lymph node follicular hyperplasia) were comparable to lymphoproliferative lung lesions reported in human immunodeficiency virus-infected children with acquired immunodeficiency syndrome. (Am J Pathol 1988, 130:80–90)

LENTIVIRUSES are etiologic agents of debilitating lymphoproliferative and lymphocytolytic disease syndromes of several animal species, including humans. Recently, human immunodeficiency virus (HIV), the etiologic agent of acquired immunodeficiency syndrome (AIDS), has been grouped with the lentiviruses on the basis of viral genome sequence homology,[1,2] viral morphology,[1] and viral replicative mechanisms.[3,4] In a strain-dependent manner, ovine lentiviruses (OvLV), when inoculated in neonatal

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lambs, will produce an accelerated form of lymphoid interstitial pneumonia (LIP) that is comparable morphologically and clinically to LIP associated with human AIDS patients.[5]

Lentivirus strains that differ in cytopathogenicity and replicative ability *in vitro* have been reported both for the ruminant lentiviruses[6,7] and the human lentivirus HIV.[8,9] Recently, ruminant lentivirus isolates have been grouped into distinct types based on lytic or nonlytic phenotype in culture, genome restriction mapping, and group-specific proteins.[10] The role of distinct virus strains in the pathogenesis of lentivirus-induced disease is not clear.

We report here that *in vitro* phenotypically distinct OvLV strains, when inoculated into neonatal lambs, result in strain-dependent patterns of lesion development. Our OvLV strains, when inoculated in neonatal lambs, fail to induce efficient virus-neutralizing serum antibodies. Anti-OvLV glycoprotein antibody is detected earlier in those lambs that develop severe OvLV-induced lesions, compared with OvLV-infected lambs with mild or no lesions, suggesting a failure of the humoral immune response to prevent lentivirus-induced lesions. Our results indicate that bronchoalveolar lavage cells from lambs with severe lymphoid interstitial pneumonia have increased numbers of a distinct T-cell subset likely to be important in the pathogenesis of lentivirus-induced lesions. Furthermore, in lambs, when inoculated with a pathogenic OvLV strain (85/34), a spectrum of pulmonary lesions develops that is similar to HIV-associated lymphoproliferative lung lesions in pediatric AIDS victims.

Materials and Methods

Cells

Monolayer cultures of goat synovial membrane cells (GSM) cells (kindly provided by Dr. D. S. Adams, Washington State University, Pullman, Wash) were used for growth of virus stocks, virus neutralization tests, virus isolation, and syncytia-induction endpoint titration assays. Goat synovial membrane cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (HI-FBS), 10 mg/ml glutamine, and 50 μg/ml gentamicin.

Virus

The growth and plaque-cloning procedures of virus stocks have been previously described.[11] Ovine lentivirus isolates were phenotypically characterized on the basis of cytopathic effects (lytic or nonlytic) when inoculated in GSM monolayers.[10] Lytic OvLV isolates (85/34 and H-24) caused fusion (syncytia) and lysis of GSM monolayers within 7–10 days after infection. Nonlytic OvLV isolates (84/28 and 85/14) produced minimal syncytia and did not cause lysis of GSM monolayers.[12] Ovine lentivirus (OvLV) strains used for animal inoculations and infection of cell cultures were isolated from either naturally occurring or experimentally induced cases of lymphoid interstitial pneumonia (LIP) (OvLV 85/34 and OvLV H-24) or were isolated from sheep with LIP and concurrent ovine pulmonary carcinoma (OvLV 84/28 and OvLV 85/14).[5,13] Virus stocks were titered with a syncytia-induction endpoint dilution assay using GSM cells. Ovine lentivirus reisolated from tissues was identified by typical cytopathic effects in GSM cells and by detection of OvLV group-specific antigen (*gag*) p26 by immunoblotting, as previously described,[13] or using an avidin–biotin peroxidase complex procedure.[14] Rabbit anti-CAEV serum, which detects OvLV *gag* p26,[10] was kindly provided by Dr. T. C. McGuire, Washington State University, Pullman, Washington.

Animal Source and Experimental Inoculation Design

Newborn Suffolk and crossbred lambs, of both sexes, were obtained prior to suckling from OvLV antibody-negative ewes. The lambs were maintained on bovine colostrum and milk replacer as previously described.[5] All lambs and adult sheep were tested for the presence of antibodies to OvLV with an agar gel immunodiffusion test with cell culture-derived ovine progressive pneumonia virus as antigen.[15] All experimental lambs were negative for OvLV serum antibodies prior to inoculation, as were control lambs throughout the study. Lentivirus-inoculated lambs were divided into two groups. All OvLV-infected lambs were inoculated intratracheally between 24 and 48 hours of age with 1×10^6 $TCID_{50}$ of virus. Group 1 lambs (n = 12) were inoculated with a low-passage, plaque-purified, lytic OvLV isolate, 85/34. Group 1 lambs (2 each time period) were sacrificed after inoculation at the following intervals: 4, 10, 16, and 24 weeks. These animals were used to evaluate early lesions of OvLV-induced LIP. Four additional lambs from this group were sacrificed at 34, 40, 41, and 43 weeks after inoculation for assessment of lesions of OvLV-induced LIP and for evaluation of immunologic parameters of OvLV-induced disease.[11] Group 2 lambs (n = 6) were inoculated with either of two low-passage, plaque-purified, nonlytic isolates, 3 lambs with OvLV 84/28 and 3 lambs with OvLV 85/14. Group 2 lambs were sacrificed between 21 and

28 weeks after inoculation for assessment of the ability of nonlytic OvLV isolates (compared with lytic isolates) to induce disease in lambs. The cell culture phenotype of all three OvLV isolates is the subject of another report.[12] Five control lambs were inoculated with virus-free GSM cell culture medium (n = 3) or were uninoculated (n = 2). Animals in assigned groups were housed within barrier-maintained isolation facilities for prevention of horizontal transmission of OvLV.

Bronchoalveolar Lavage

Bronchoalveolar lavage was performed in lambs immediately after death with sterile Hanks' balanced salt solution (HBSS, pH 7.4) supplemented with 0.5 μg/ml amphotericin B and 50 μg/ml gentamicin (Irvine Scientific, Santa Ana, Calif) as previously described.[5] The proportion of viable cells, assessed by trypan blue dye exclusion, was determined by hemocytometer counts. Bronchoalveolar lavage cells (BACs) were either used for virus isolation or cytocentrifuged onto glass slides. Alveolar macrophages (AMs) were identified by characteristic morphology (Wright's stain) and by either neutral red dye uptake or latex bead phagocytosis.[16] In addition, AMs failed with react with monoclonal antibodies against ovine T cells (below).

Identification of Lymphocyte Phenotypes

Lymphocytes were identified in BAC by means of murine monoclonal antibodies (MAbs) specific for ovine lymphocytes as described.[17-19] These MAbs were kindly supplied by Drs. C. R. Mackay and M. R. Brandon, University of Melbourne, Parkville, Victoria, Australia. Briefly, cytocentrifuge preparations of BAC were fixed in either acetone or methanol for 15 minutes and stored at −70 C. Cytocentrifuge preparations used in MAb procedures were placed in phosphate-buffered saline (PBS, pH 7.4) for 5 minutes before being incubated for 20 minutes in 0.3% hydrogen peroxide (H_2O_2, diluted in methanol) to inactivate endogenous peroxidase activity. After washing (three times in PBS), 80 μl of primary MAbs diluted in PBS (1 : 10 dilution of high-titer MAb cell culture supernatant) was placed on the cells and incubated in a humidified chamber at 37 C for 60 minutes. The preparations were then rinsed in PBS and incubated with 80 μl of biotinylated horse anti-mouse IgG antibody solution (Vector, Burlingame, Calif) as with primary antibody steps. After rinsing with PBS, 80 μl of an avidin–biotinylated horseradish peroxidase solution (ABC) (Vector, Burlingame, Calif) was incubated with the cells as with primary antibody steps. Color reaction product was developed with the chromogen 3,3′-diaminobenzidine (Sigma, St. Louis, Mo). The slides were counterstained with a 1.0% solution of methyl green in distilled water, coverslipped, and examined. The specificity of the immunostaining was determined by omitting the primary antibody step, using nonspecific MAbs, and applying only the ABC and substrate steps. Differences between groups, in a comparison of numbers of SBU-$T1^+$ (pan T cell), SBU-$T8^+$ (CD 8^+ equivalent, cytotoxic/suppressor), SBU-II^+ (major histocompatible complex, [MHC] Class II antigen equivalent) cells and total lymphocytes, were statistically determined by means of one-way analysis of variance. *P* values of less than 0.05 were considered significant, and *P* values of between 0.1 and 0.05 were considered less significant, but biologically relevant.

Serology, Hematology, and Clinical Evaluation

Identification of serum precipitating antibodies was performed with an agar gel immunodiffusion test (AGID) with cell culture-derived ovine progressive pneumonia virus as antigen.[15] Precipitation lines of identity and nonidentity with ovine anti-OvLV antiserum (kindly provided by Dr. R. C. Cutlip, National Animal Disease Laboratory, Ames, Iowa) were determined. Lines of nonidentity were indicative of antibodies to OvLV core protein p26, and lines of identity were indicative of antibodies to OvLV envelope glycoprotein gp135.[13]

Ovine lentivirus neutralizing antibodies were detected from serum samples using GSM cells in a modification of a previously described procedure.[20] Briefly, OvLV (1000 $TCID_{50}$) was incubated with serial twofold dilutions of test serum in DMEM plus 5% HI-FBS (previously shown not to inhibit OvLV replication) and 50 μg/ml gentamicin and incubated for 60 minutes at 37 C, then overnight at 4 C. Four replicate wells of confluent layers of GSM cells in a 96-well microtiter plate were inoculated with each dilution of virus plus test serum (100 μl/well) and incubated 11 days in a humidified chamber at 37 C before being fixed in methanol, stained (Giemsa), and evaluated for cytopathic effects of OvLV. Virus-neutralizing titers were considered the highest reciprocal dilution at which OvLV cytopathic effects were prevented in at least 50% of the inoculated wells.

The inoculated lambs were monitored daily for clinical signs of respiratory disease. Hematologic parameters (leukocyte and erythrocyte counts, hematocrit, and leukocyte differential counts) were monitored at weekly intervals (through 4 weeks of age) and

at monthly intervals (until death or through 6 months of age).

Gross and Histologic Assessment of Lesions

Lambs were anesthetized by intravenous inoculation of xylazine and ketamine and killed by exsanguination. Selected organs were excised and weighed. Tissues were fixed in 10% buffered neutral formalin solution, sectioned at 5 μ, and stained with hematoxylin and eosin (H&E). After bronchoalveolar lavage, the left lung lobe was excised and insufflated with 10% buffered neutral formalin solution at 30 cm of water pressure for 48 hours before sectioning for histologic examination. Lymphoid interstitial pneumonia was defined and ranked as previously described.[5]

Virus Isolation

Tissues and cells for virus isolation were collected at the time of death and cocultured with GSM cells as previously described.[5] Peripheral blood mononuclear cells (PBMCs) were isolated from blood with Ficoll-Hypaque (specific gravity 1.077) for assessment of viremia as previously described.[16] Selected lung samples were cultured for *Mycoplasma* and aerobic bacterial species and were also examined by fluorescent antibody techniques for the presence of antigens of bovine respiratory syncytial virus and bovine virus diarrhea virus.[5]

Results

Clinical Signs and Lesions

Clinical signs of respiratory distress or lameness were limited to those lambs inoculated with lytic OvLV 85/34, Group 1 (Table 1). Five of 12 lambs of Group 1 had episodes of increased resting respiratory rates or dyspnea, beginning as early as 3 weeks after inoculation (5 and 15) or developing near the time of sacrifice (25, 26, and 37). All 5 lambs with respiratory distress had moderate to severe lesions of lymphoid interstitial pneumonia and/or pulmonary lymphoid hyperplasia; however, 3 additional lambs from this group (79, 36, and 24) with mild lesions of OvLV-LIP did not have observable respiratory distress (Table 1). One lamb (36) developed right carpal swelling at 32 weeks after inoculation (Table 1), which progressed to

Table 1—Ovine Lentivirus Infection of Lambs: Serum Antibody, Clinical Signs, and Lesions

Lamb (inoculum)*	Sacrifice (weeks)	Serum antibody (wk+)† AGID p28	AGID gp135	VNT	Clinical signs‡ Dyspnea	Clinical signs‡ Arthritis	Lesions§ LIP	Lesions§ LN	Lesions§ MG	Lesions§ J
Group 1 (85/34)										
5	4	3	4	nd	++	–	+++	++	–	–
79	4	–	–	nd	–	–	+	–	–	–
13	10	–	–	nd	–	–	–	–	–	–
15	10	4	5	–	++	–	+++	++	M	–
26	16	4	6	–	++	–	++++	++	–	nd
37	16	–	8	1:10	+	–	+++	++++	++	nd
23	24	5	9	–	–	–	–	–	M	–
27	24	4	15	1:10	–	–	–	–	–	–
38	34	5	8	1:10	–	–	–	+	M	++
36	40	4	13	1:10	–	++	+	–	–	+++
24	41	4	5	–	–	–	+	–	+	–
25	43	4	6	–	+	–	++++	++++	+++	+
Total		9/12	10/12	4/9	5/12	1/12	8/12	6/12	3/9	3/10
Group 2										
28 (85/14)	24	4	16	–	–	–	+	++	M	–
29 (85/14)	24	–	–	1:40	–	–	–	–	–	–
43 (85/14)	28	3	–	–	–	–	–	–	M	–
33 (84/28)	24	–	5	–	–	–	+	–	M	–
34 (84/28)	24	3	11	–	–	–	–	–	M	–
46 (84/28)	21	6	20	–	–	–	+	+	–	–
Total		4/6	4/6	1/6	0/6	0/6	3/6	2/6	0/2	0/6

*Inoculum OvLV isolates as in Materials and Methods. Five control lambs inoculated with media (n = 3) or uninoculated (n = 2), killed between 16 to 35 weeks of age, did not have clinical signs, lesions, or OvLV antibodies.

†OvLV serum antibody (time in weeks after inoculation of detection of antibody) to *gag* p26 and *env* gp135 detected by the agar gel immunodiffusion test (AGID). Virus neutralization test (VNT) using serum at sacrifice and tested against the OvLV strain against which the lamb was inoculated.

‡Dsypnea: +, mild increase in respiratory rate; ++, increased resting respiratory rate with exercise intolerance.

§Lesions: LIP, lymphoid interstitial pneumonia ranked as previously described[6]; LN, pulmonary lymph node follicular hyperplasia; MG, mammary gland-lymphocytic mastitis; J, joint-lymphocytic synovitis; no lesion; +, mild lesion; ++++, severe lesion; M, male lamb, mammary gland not assessed.

a firm, nodular enlargement of the entire joint and periarticular tissue. No clinical signs of respiratory disease or lameness were observed in Group 2 lambs (inoculated with nonlytic OvLV isolates) or Group 3 lambs (controls) throughout the study.

At necropsy, gross lesions were limited to 6 of 12 lambs in Group 1. Multifocal minute to 3.0-mm raised gray and tan foci marked the pleural surface of 3 of these lambs in Group 1 (79, 15, and 24). Three additional lambs within this group had larger (1.0 to 3.0 cm in diameter) raised gray to tan nodules. On the sectioned surface, these nodules extended into lung parenchyma at pleural surfaces and also were located adjacent to main bronchi.

The average lung weight of Group 1 lambs with moderate to severe lesions (5, 15, 25, 26, and 37), expressed as a percentage of body weight (%BW), was 2.1% BW, compared with 1.0% BW of the 5 control lambs. Group 1 lambs with moderate to severe lesions of LIP also had enlarged caudal mediastinal lymph nodes (primary pulmonary lymph node) weighing 0.032% BW, compared with 0.014% BW of the control lambs. Lamb 25 of Group 1 had swollen, edematous mammary gland tissue due to severe lymphoproliferative mastitis (see below). The right carpus of Lamb 36 from Group 1 had swollen, proliferative and edematous periarticular tissue with swollen, tan and edematous synovium. Lambs inoculated with nonlytic isolates of OvLV (Group 2) and control lambs had no gross lesions.

Microscopic lesions of lymphoid interstitial pneumonia and/or pulmonary lymphoid hyperplasia were present in 8 of 12 lambs inoculated with the low-passage, lytic OvLV isolate 85/34 (Table 1). Mild lesions consisted of multifocal peribronchiolar and perivascular lymphoid follicles with minimal interstitial infiltrates (24, 36, and 79). These follicles were composed of medium to large lymphocytes with occasional centrally located and mitotically active germinal centers. The lymphoid follicles often compressed bronchiolar lumens, and lymphocytes were observed between viable bronchiolar epithelial cells and in the lumens of bronchioles (Figure 1).

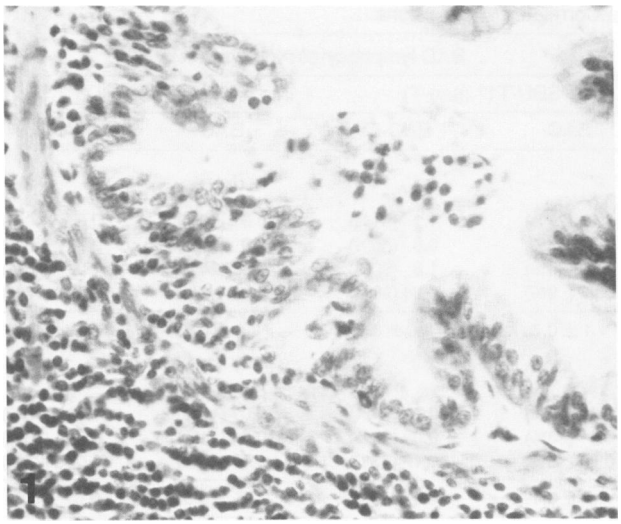
More severe LIP lesions consisted of two principal morphologic patterns: large, multifocal to confluent irregular aggregates of lymphocytes (pulmonary lymphoid hyperplasia) and interstitial pneumonitis. Large subpleural nodules observed grossly within LIP-affected lambs consisted of sheets of medium to large lymphocytes. These lymphoid aggregates were often confluent, with interstitial septa that were severely thickened by heterogeneous inflammatory infiltrates (lymphocytes, macrophages, plasma cells, and rarely eosinophils) (Figure 2). Ultrastructural examination of interstitial infiltrates of Lamb 15 with 3+ LIP demonstrated the lymphocytic composition of the interstitial lesion (Figure 3). Lesions of pneumonitis often had alveoli which were lined by prominent Type II pneumocytes (Figure 3). Large pleomorphic macrophages, fibrin, lymphocytes and occasionally eosinophils were within alveoli.

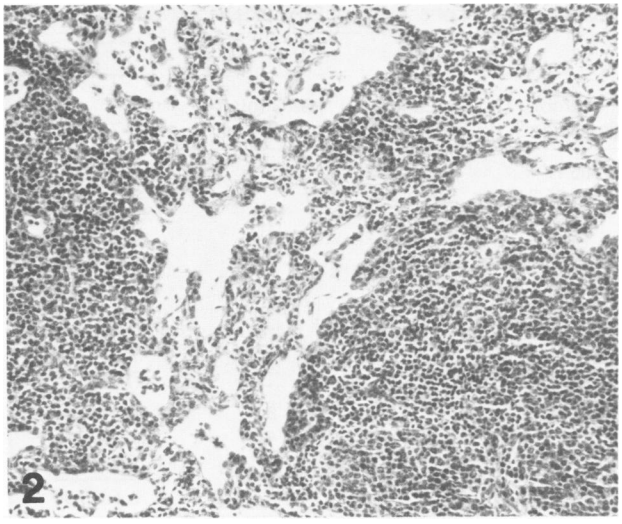
In 3 of 6 Group 2 lambs, inoculated with nonlytic OvLV isolates, LIP lesions developed. In contrast to Group 1 lambs, the lesions in Group 2 lambs were all mild (1+). Group 2 lambs with lesions (28, 33, and 46) had minimal interstitial infiltrates, and the extensive lymphoid hyperplastic lesions did not develop, as observed in Group 1 lambs (Table 1). In control lambs microscopic lesions did not develop.

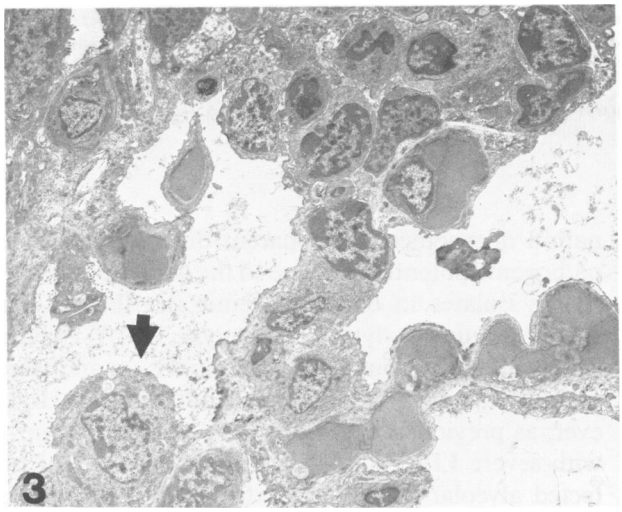
Bronchoalveolar lavage samples for OvLV lambs confirmed previously reported findings[5] that the development of OvLV-induced LIP is accompanied by increased numbers and percentages of lymphocytes (Table 2). To further assess the phenotype of infiltrative or proliferative lymphocytes from the lung, we examined BACs with monoclonal antibodies against lymphocyte surfaces antigens. As shown in Table 3, 3 of 4 Group 1 lambs examined (24, 25, and 36) had significantly increased total numbers ($P<0.05$) and increased percentages of T cells in BAC samples; 1.2–2.0 $\times 10^5$ total T cells (80–91% of total lymphocytes), compared with 0.1–0.3 $\times 10^5$ total T cells (38–63% of total lymphocytes) for control Lambs 35, 42, and 47 (Table 3).

The predominant lymphocyte subset in BAC samples of the 3 OvLV-diseased lambs (24, 25, and 36) was of SBU-T8$^+$ phenotype (49–75% of total lymphocytes) (Figure 4, Table 3). The total number of SBU-T8$^+$ lymphocytes of these OvLV-infected lambs was also significantly greater ($P<0.05$) than the total number of SBU-T8$^+$ cells in BAC samples of control lambs (Table 3). The percentage of SBU-T8$^+$ lymphocytes of control lamb BAC samples did not exceed 25% of total BAC lymphocytes. In addition, Lamb 38 (which did not have significant OvLV-induced disease) had similar percentages of total lymphocytes and SBU-T8$^+$ lymphocytes, compared with control lamb BAC samples (Table 3). On average, the proportion of BACs expressing SBU-II (MHC Class II equivalent) in OvLV-infected lambs (14.1% ± 5.1%, n = 4) was not significantly different ($P>0.1$) from that of similar samples of control lambs (11.6% ± 7.3, n = 3).

Pulmonary lymph node follicular hyperplasia correlated with the degree of lung lesions (Table 1). Group 1 lambs with severe LIP lesions (5, 15, 25, 26, and 37) had 39.1 ± 16.2 mean germinal centers per lymph node cross-section, compared with 14.5 ± 6.0







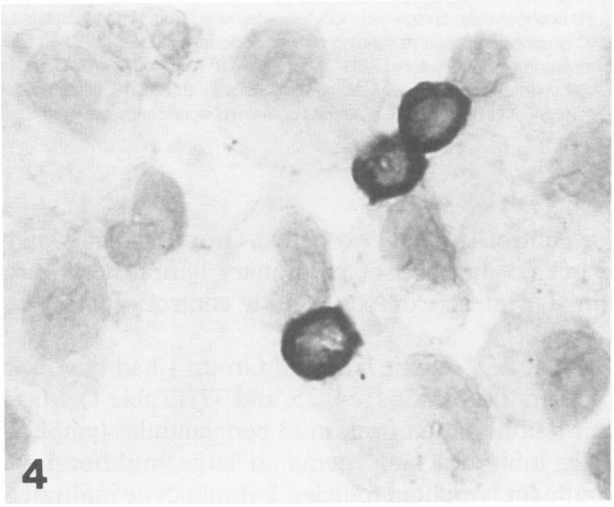


Figure 1—Lymphoid follicle adjacent to a bronchiole with lymphocytes infiltrating between epithelial cells and within the bronchiolar lumen. (H&E, ×320) **Figure 2**—Section of lung from an OvLV-inoculated lamb (25, Group 1) with irregular aggregates of lymphocytes (pulmonary lymphoid hyperplasia) adjacent to interstitium thickened by heterogeneous inflammatory cell infiltrates. (H&E, ×160) **Figure 3**—Electron micrograph of pulmonary interstitial infiltrates from an OvLV-inoculated lamb (15, Group 1). Medium-sized lymphocytes (*upper right*) within thickened septum, adjacent alveoli with protruding Type II cell (*arrow*). (Uranyl acetate and lead citrate, ×2800) **Figure 4**—Cytocentrifuge preparation of bronchoalveolar lavage sample of OvLV-inoculated lamb (36, Group 1). Three SBU-T8 monoclonal antibody-positive lymphocytes adjacent to alveolar macrophages. (ABC with methyl green, ×1400)

Table 2—Bronchoalveolar Lavage Cells of Lambs Correlated With Lung Lesions

Lamb (inoculum)	Lung lesion rank†	Bronchoalveolar lavage cells (× 10^5/ml)‡		
		Total cells	Alveolar macrophages	Lymphocytes
Group 1 (n = 12) (OvLV 85/34)	1.7	5.8 ± 4.6	4.3 ± 3.1	1.3 ± 1.2§
Group 2 (n = 6) (OvLV 84/28 and 85/14)	0.5	3.4 ± 1.4	3.0 ± 1.4	0.7 ± 0.9
Group 3 (n = 5) (Controls)	0	3.7 ± 1.5	3.4 ± 1.5	0.3 ± 0.1

*Inoculum OvLV isolates inoculated as in Materials and Methods. Controls were inoculated with medium (n = 3) or were left uninoculated (n = 2).

†Mean lesion rank as previously described[5]; 0, no lesions; 4+, severe lesions.

‡BACs collected at necropsy, expressed as mean number ± SD × 10^5 cells/ml of recovered fluid, 500–1000 ml lavage fluid instilled in each lamb's lung, with average recovery of 50–75% of instilled fluid. BAC differential count determined from Wright's stained cytocentrifuge preparations. Only alveolar macrophages and lymphocytes are shown in the table; the range of other cell types (neutrophils and eosinophils) was 1–9% of total BACs.

§Significantly different from controls ($P < 0.01$).

Table 3—Immunophenotype of Bronchoalveolar Lavage Lymphocytes Correlated With Lesions

Lamb (inoculum*)	Lung lesion	BAC total† $\times 10^5$/ml	BAC lymphocytes $\times 10^5$/ml (%)‡				
			Total	SBU-T1$^+$ (pan-T)		SBU-T8$^+$ (CD8$^+$)	
				BAC	BAC-LY	BAC	BAC-LY
Group 1 (OvLV 85/34)							
24§	+	5.6	1.3 (23%)	1.2 (21%)	1.1 (91%)	0.6 (10%)	0.3 (49%)
25§	++++	6.0	1.2 (20%)	1.0 (16%)	0.8 (80%)	0.9 (15%)	0.7 (75%)
36§	+	6.8	2.2 (33%)	2.0 (30%)	1.8 (90%)	1.6 (24%)	1.2 (73%)
38	−	2.8	0.3 (10%)	0.27 (9%)	0.24 (90%)	0.03 (1%)	0.003 (10%)
Totals ± SD			1.3 ± 0.8	1.1 ± 0.7	1.0 ± 0.6	0.8 ± 0.6	0.6 ± 0.5
Group 3 (controls)							
35	−	3.7	0.4 (11%)	0.2 (6%)	0.11 (54%)	0.07 (2%)	0.01 (18%)
42	−	4.5	0.4 (8%)	0.1 (3%)	0.04 (38%)	0.04 (1%)	0.005 (13%)
47	−	5.7	0.5 (8%)	0.3 (5%)	0.19 (63%)	0.11 (2%)	0.028 (25%)
Totals ± SD			0.4 ± 0.06	0.2 ± 0.1	0.1 ± 0.08	0.07 ± 0.04	0.01 ± 0.01

*Inoculum as in Materials and Methods.

†Bronchoalveolar lavage cells, 10^5/ml in recovered lavage fluid, 1000 ml instilled with mean recovery of 70% of instilled fluid.

‡Bronchoalveolar lavage lymphocytes determined from Wright's stained cytocentrifuge preparations and hemocytometer counts. SBU-T1$^+$ and SBU-T8$^+$, BAC lymphocytes positive staining with monoclonal antibodies SBU-T1 (pan-T-cell) and SBU-T8 (CD8 equivalent), respectively, with the ABC method, using cytocentrifuge preparations. SBU-T1$^+$ or SBU-T8$^+$ lymphocytes/BAC, total and percent MAb-positive cells in total BACs. SBU-T1$^+$ or SBU-T8$^+$ lymphocytes/BAC-Ly, total and percent MAb-positive cells in total BAC lymphocytes.

§Lambs of Group 1 with LIP lesions contained significantly elevated total SBU-T1$^+$ ($P < 0.05$) and total SBU-T8$^+$ cells ($P < 0.05$), compared with controls.

for control lambs. Two of the Group 2 lambs had increased numbers of pulmonary lymph node germinal centers, compared with controls (data not shown).

Three of 9 female lambs in Group 1 had lymphoproliferative mastitis (24, 25, and 37) (Table 1). Mastitis lesions varied from mild periglandular lymphocytic infiltrates and edema to large multifocal to confluent lymphoid follicles. Lymphocytic infiltrates in mastitis lesions of Lambs 25 and 37 compressed adjacent glandular and ductular mammary gland tissue (Figure 5).

Lambs 25, 36, and 38 of Group 1 had lymphocytic and plasmacytic infiltrates in carpal joint synovial membranes (Table 1). These synovial lesions consisted of plasma cell infiltrates surrounding blood vessels lined by prominent endothelial cells (Figure 6). Synovial villi were enlarged by edema, fibrin, and moderate numbers of macrophages (occasionally multinucleate). Group 2 and control lambs did not have any systemic lymphoproliferative lesions. All hematologic parameters for both OvLV-infected groups were within the range of controls throughout the study.

Lesion data indicated that lytic OvLV isolate 85/34, while not inducing lesions in all inoculated lambs, was capable of producing severe and rapid lesions of the lung, mammary gland, and synovium. Tissue virus isolation data was assessed for determination of whether this apparent difference in virus pathogenic potential was due to the ability of the lentivirus isolates to obtain systemic distribution. As shown (Figure 7), lytic and nonlytic OvLV isolates were widely distributed in systemic tissues and were present in peripheral blood mononuclear cells. However, as previously reported,[11] lambs from Group 1 with severe LIP lesions had greater numbers of infected alveolar macrophages from bronchoalveolar lavage samples. In addition, the percentage of tissues positive by virus isolation was greater in Group 1 lambs (Figure 7), compared with Group 2 lambs. Selected lung samples from OvLV-infected lambs were negative when cultured for *Mycoplasma* species or other opportunistic bacterial species and were negative for the presence of bovine respiratory syncytial virus or bovine virus diarrhea virus antigens.

In both Group 1 and Group 2 lambs precipitating antibodies to ovine lentivirus developed (Table 1). However, on average, Group 1 lambs that had severe lung lesions produced serum precipitating antibodies against OvLV envelope gp135 earlier than Group 1 lambs that had mild or no lung lesions (5.8 weeks for Lambs 5, 15, 26, 25, and 37 versus 10.2 weeks for lambs 23, 24, 27, 36, and 38). In addition, Group 2 lambs inoculated with nonlytic OvLV isolates that formed anti-gp135 precipitating serum antibodies (4 of 6) did so later than Group 1 lambs with severe LIP lesions (average, 13 weeks for Group 2 Lambs 28, 33,

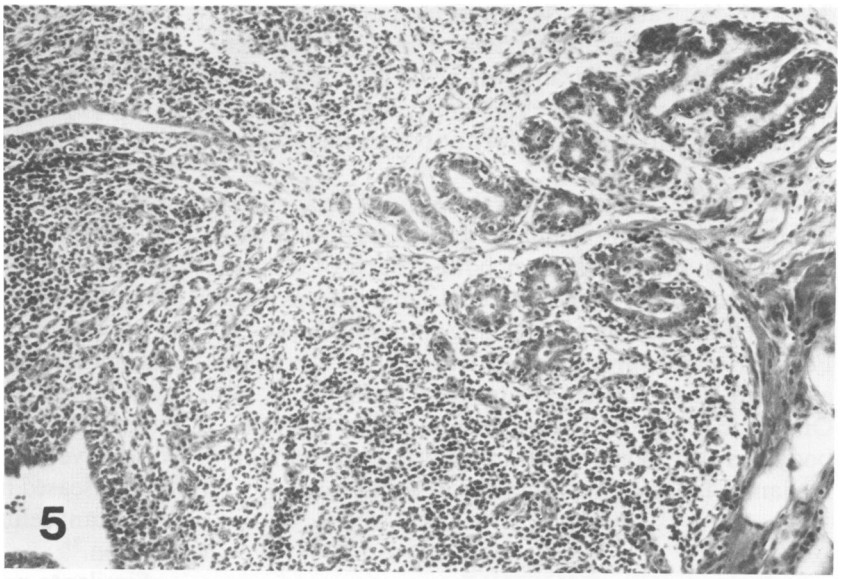


Figure 5—Section of mammary gland from an OvLV-inoculated lamb (25, Group 1) with severe lymphocytic infiltrates surrounding mammary gland acini. (H&E, ×120)

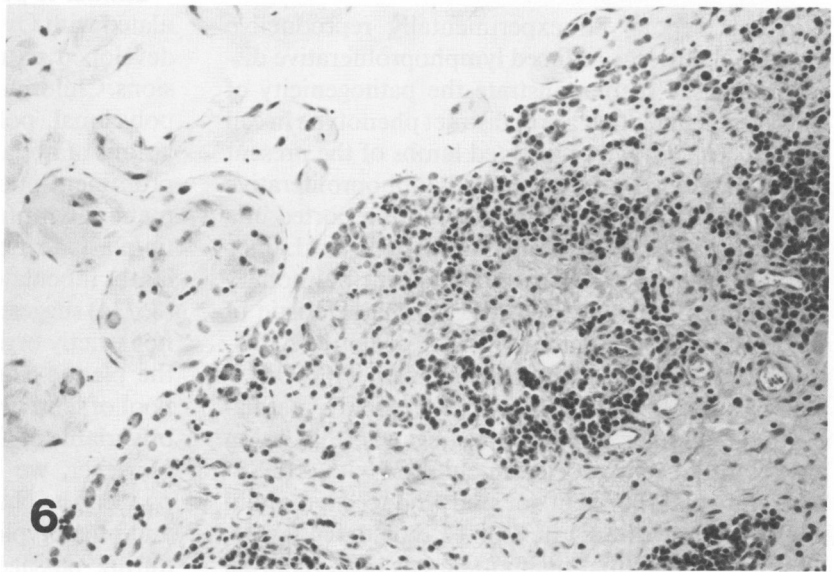


Figure 6—Synovium of an OvLV-inoculated lamb (36, Group 1) with perivascular plasma cell and macrophage infiltrates. (H&E, ×185)

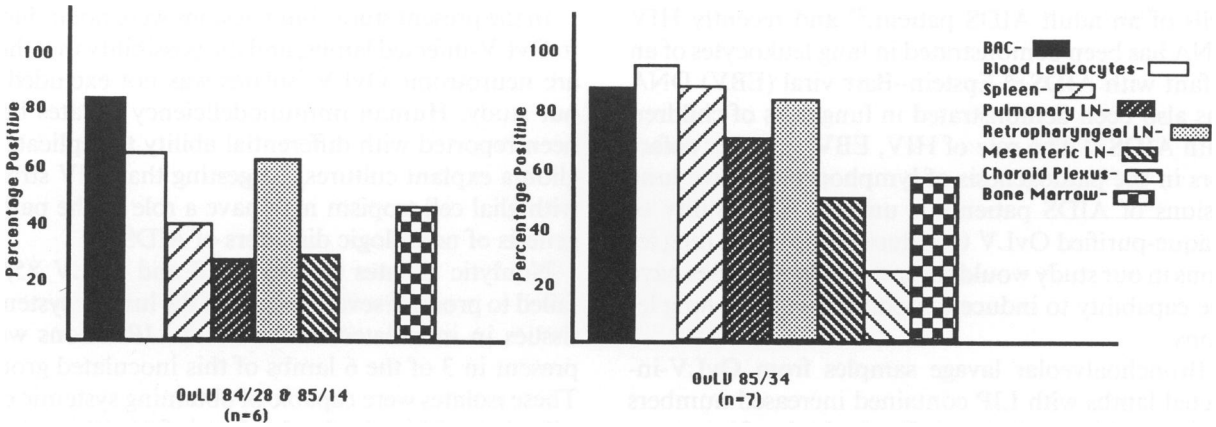


Figure 7—Lentivirus tissue distribution in OvLV-inoculated lambs. Summary of OvLV isolation results of 7 lambs tested from Group 1 (inoculated with OvLV 85/34) and 6 lambs tested from Group 2 (inoculated with OvLV 84/28 or 85/14).

34, and 46, Table 1). Serum from lambs of both OvLV-inoculated groups produced early (3–6 weeks after inoculation) and transient lines of nonidentity to lentivirus antigen. These nonidentity lines were previously shown to be specific for antibodies against *gag* p28 of OvLV.[13]

To further assess humoral immune responses against lentivirus, we evaluated serum OvLV-neutralizing (VN) antibodies from both groups of lambs. Serum from both OvLV-infected lamb groups had low levels or no virus-neutralizing antibody (Table 1). Four of 9 lambs from Group 1 had VN antibodies, although never at titers greater than 1 : 10 (Table 1). One lamb (29) from Group 2 had a serum VN antibody titer of 1 : 40; however, serum from the remaining lambs failed to neutralize ovine lentivirus.

Discussion

In this report, an experimentally reproducible model of lentivirus-induced lymphoproliferative disease was used to demonstrate the pathogenicity of lentivirus isolates that are of distinct phenotype in cell culture. The lentivirus-infected lambs of the present report developed a spectrum of lymphoproliferative pulmonary lesions that have also been reported in a high percentage of pediatric AIDS patients. Lambs with severe lung lesions had concurrent pulmonary lymph node follicular hyperplasia, characteristic of OvLV-induced pneumonia[5,21] and pediatric AIDS-associated LIP.[22,23] Lambs inoculated with OvLV 85/34 also developed pulmonary lymphoid hyperplasia (large nodular lymphoid aggregates); this lesion has recently been reported in children with AIDS.[24] The lytic isolate 85/34 in our study induced lymphoid interstitial pneumonia in 8 of 12 inoculated lambs. Lymphoid interstitial pneumonia has been reported as a frequent sequela in pediatric AIDS victims.[22] HIV has been cultured from bronchoalveolar lavage cells of an adult AIDS patient,[25] and recently HIV RNA has been demonstrated in lung leukocytes of an infant with AIDS.[26] Epstein–Barr viral (EBV) DNA has also been demonstrated in lung cells of children with AIDS.[27] The role of HIV, EBV, or other cofactors in the pathogenesis of lymphoproliferative lung lesions of AIDS patients is unclear. The ability of plaque-purified OvLV to induce comparable lung lesions in our study would suggest that lentiviruses have the capability to induce lymphoproliferative lung lesions.

Bronchoalveolar lavage samples from OvLV-infected lambs with LIP contained increased numbers of SBU-T8$^+$ lymphocytes. On the basis of immunocytochemical and biochemical data, this monoclonal antibody is considered the structural analog of marker molecules used to define T-cytotoxic/suppressor lymphocytes of human and murine lymphocyte identification systems.[18] Lymphocytes with phenotypic characteristics of cytotoxic/suppressor (T8$^+$) markers have been demonstrated in cutaneous exanthem lesions associated with primate lentivirus (simian immunodeficiency virus)[28] and HIV-associated pediatric LIP lesions.[29] To our knowledge, the present study is the first characterization of the immunophenotype of lymphocytes in OvLV-induced lymphoid interstitial pneumonia. The role of this subset of lymphocytes within lesion sites of lentivirus-induced disease is unclear. It has been proposed that T8$^+$ lymphocytes may serve as effector cells causing tissue damage in HIV-diseased humans[30] or alternatively serving as an important cellular mechanism in inhibiting HIV replication.[31]

Similar to pediatric AIDS patients, the lambs inoculated with OvLV isolate 85/34 in the present report developed extrapulmonary lymphoproliferative lesions. Children with AIDS have been reported to have polyclonal, polymorphic B-cell lymphoproliferative lesions of nodal and extranodal sites with concurrent prominent pulmonary involvement.[24] The development of lymphoproliferative mastitis and lymphocytic/plasmacytic synovitis, concurrent with LIP, in lambs inoculated with a plaque-cloned OvLV isolate (85/34) suggests that pathogenic lentivirus strains are not strictly organ-specific in their effects. Less likely, the plaque-cloned isolate may consist of a limited pool of lentiviruses with similar *in vitro* phenotypes, or variants of OvLV may have arisen during passage. However, we have subsequently repeated and expanded the characterization of our OvLV isolates,[12] and phenotypic characteristics have remained stable during passage. Genome and antigenic structural comparisons of the OvLV isolates used in this report are currently being investigated.

In the present study, brain lesions were not induced in OvLV-infected lambs, and the possibility that there are neurotropic OvLV isolates was not excluded in our study. Human immunodeficiency isolates have been reported with differential ability to replicate in glioma explant cultures, suggesting that HIV strains with glial cell tropism may have a role in the pathogenesis of neurologic disorders of AIDS.[9]

Nonlytic isolates OvLV 84/28 and OvLV 85/14 failed to produce severe lesions of the lung or systemic tissues in inoculated lambs. Mild LIP lesions were present in 3 of the 6 lambs of this inoculated group. These isolates were capable of obtaining systemic distribution within the lambs, but infected lower percentages of alveolar macrophages.[11] In another re-

port, we showed that these isolates replicated less efficiently in and caused less fusion and lysis of alveolar macrophages.[12] Taken together, these data suggest that pathogenic OvLV isolates may cause accelerated lymphoproliferative disease in neonatal lambs as a consequence of effective and lytic replication in alveolar macrophages.

Lambs that developed severe lymphoproliferative lesions produced precipitating antibodies to lentivirus earlier, compared with virus-inoculated lambs that had either mild or no lesions. The earlier production of antibodies may be due to enhanced replication of the lytic OvLV isolate, allowing greater exposure of viral antigen for immune detection. Alternatively, early onset of nonneutralizing OvLV antibodies may enhance virus replication in these lambs through antibody-enhanced infection of macrophages.[32] In either case, antibodies produced by the lambs, with or without disease, had either limited or no capacity to neutralize OvLV *in vitro*, which indicates that humoral immune responses may not play an important role in protecting lentivirus-infected animals.

It has been proposed that lytic OvLV isolates evoke rapid immune response against viral glycoprotein and subsequent high neutralization titers, whereas nonlytic OvLV strains induce less consistent responses to glycoprotein.[33] Our study demonstrated that lytic and nonlytic OvLV isolates induce similar responses to viral glycoprotein (however, antibody responses to the nonlytic isolates were slower in onset) and that induction of virus neutralizing antibody is not related to the ability of lentivirus isolates to cause lysis of cultured cells. The failure to produce VN antibodies was not related to immune suppression of the lambs[11] or, as demonstrated in the present investigation, to the ability of the virus isolates to replicate within the host. Failure of the host to form active neutralizing antibody, while concurrently producing nonneutralizing precipitating antibodies, is more likely related to specific structural features of the viral envelope which inhibit VN antibody induction. Alternatively, failure to form virus neutralizing antibodies may be due to blocking of protective antibodies by nonneutralizing antibodies or a specific inability of the host to respond or recognize important epitopes that will prevent lentivirus infection.

This study demonstrated that ovine lentivirus isolates induced strain-dependent patterns of lesion development. Pulmonary inoculation of a plaque-cloned OvLV caused lymphoproliferative lesions of the lung, mammary gland, and synovium, suggesting that lentivirus strains are not strictly organ specific in pathogenicity. Analysis of bronchoalveolar lavage cell samples suggest that SBU-T8$^+$ lymphocytes may have a role in lesion development in lentivirus-diseased lambs. Neonatal lambs inoculated with defined OvLV isolates can provide a reproducible model for further investigation of the role of viral and host factors in lentivirus-induced disease.

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