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Pathogenicity of Molecularly Cloned Bovine Leukemia Virus

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To delineate the mechanisms of bovine leukemia virus (BLV) pathogenesis, four full-length BLV clones, 1, 8, 9, and 13, derived from the transformed cell line FLK-BLV and a clone construct, pBLV913, were introduced into bovine spleen cells by microinjection. Microinjected cells exhibited cytopathic effects and produced BLV p24 and gp51 antigens and infectious virus. The construct, pBLV913, was selected for infection of two sheep by inoculation of microinjected cells. After 15 months, peripheral blood mononuclear cells from these sheep served as inocula for the transfer of infection to four additional sheep. All six infected sheep seroconverted to BLV and had detectable BLV DNA in peripheral blood mononuclear cells after amplification by polymerase chain reaction. Four of the six sheep developed altered B/T-lymphocyte ratios between 33 and 53 months postinfection. One sheep died of unrelated causes, and one remained hematologically normal. Two of the affected sheep developed B lymphocytosis comparable to that observed in animals inoculated with peripheral blood mononuclear cells from BLV-infected cattle. This expanded B-lymphocyte population was characterized by elevated expression of B-cell surface markers, spontaneous blastogenesis, virus expression in vitro, and increased, polyclonally integrated provirus. One of these two sheep developed lymphocytic leukemia-lymphoma at 57 months postinfection. Leukemic cells had the same phenotype and harbored a single, monoclonally integrated provirus but produced no virus after in vitro cultivation. The range in clinical response to in vivo infection with cloned BLV suggests an important role for host immune response in the progression of virus replication and pathogenesis.

Bovine leukemia virus (BLV) is a B-cell lymphotropic retrovirus closely related by genomic organization and disease progression to human T-cell leukemia (lymphotropic) virus type I (21, 46, 47, 52). BLV is an important pathogen of cattle, with rates of infection as high as 50% in U.S. dairy herds (8). Infection has been associated with reduced productivity, reproductive capacity, and survival rate because of the immune disturbances associated with disease progression (5). BLV causes a nonneoplastic, persistent lymphocytosis (PL) in about 30% of infected cattle, and a neoplastic proliferation of B lymphocytes, enzootic bovine leukosis, in approximately 1% of infected animals after a 5- to 10-year latent period (19). Sheep are susceptible to experimental infection with BLV and have an increased rate and frequency of lymphoma (17, 22), frequently preceded by lymphocytosis (14, 16).

BLV-infected animals develop a persistent, high-titered antibody response to all viral proteins without apparent viremia (12). This suggests that viral antigens are presented to B cells in an efficient manner (25, 50). The B cells of animals with PL are activated, cycling cells with elevated levels of surface immunoglobulin (sIg) and class II major histocompatibility complex antigens (MHC II) (20, 40). Peripheral blood mononuclear cells (PBMC) of animals with PL have increased rates of spontaneous DNA synthesis (41, 60). BLV expression occurs rapidly following in vitro incubation of infected PBMC and is enhanced by mitogenic stimulation, providing further evidence that B-cell activation is associated with viral replication and that it may play a role in disease progression (2, 16, 19, 33, 35, 44).

The BLV provirus contains a regulatory X region, located between the *env* region and the 3′ long terminal repeat (LTR) (47, 51) with several open reading frames. Two regions encode the Tax protein, a *trans*-acting transcriptional activator, and the Rex protein, a promoter of viral structural proteins (9, 47, 48, 51). Virus transcription in vivo is severely restricted throughout infection (33, 35), but amplification of reverse-transcribed mRNA has been used to detect viral transcripts (31). The *tax/rex* mRNA is the only message consistently present at all stages of disease (24). Two additional BLV mRNAs, corresponding to X-region open reading frames, XBL-III and XBL-IV, have been identified in low abundance in infected animals (1). BLV regulatory proteins are important to virus replication and may have a role in tumorigenesis.

Molecular clones of BLV have been shown to induce syncytia and produce infectious virus in vitro (30) and have been transfected directly into sheep to establish infection (57, 58). BLV from experimentally infected fetal lamb kidney cells (FLK-BLV) (55) has been used to infect and to induce lymphosarcoma in sheep (29). The four full-length BLV proviruses in FLK-BLV cells (designated 1, 8, 9, and 13) have been cloned and used to characterize the BLV LTR as a cell type-specific promoter, active only in productively infected cells (11). Transcriptional utilizations of the LTRs of the four clones were similar. A plasmid subclone, pBLV913, constructed from clones 9 and 13, has been used for the delineation of BLV *trans*-acting factors (9, 10).

In the current study, we have characterized the activity of the previously cloned FLK-BLV proviruses and pBLV913 in vitro.

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TABLE 1. Summary of in vivo infections

Sheep no.	Mo p.i.	Inoculum
41, 84	57	BESP-913 cells
L1, L2	42	PBMC from sheep 41
L3, L4	42	PBMC from sheep 84
3, 5	70	PBMC from BLV^+ cow with PL
94	44	Uninfected bovine adherent PBMC

Further, pBLV913 was used to infect sheep and induce disease. This model provides a system to elucidate differences in the viral genome versus the host response which could be specifically associated with disease induction.

MATERIALS AND METHODS

Cell culture. FLK-BLV cells and primary bovine embryonic spleen (BESP) cells were cultured in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum and L-glutamine.

Preparation of molecular clones. The isolation of full-length BLV clones from FLK-BLV cells was described previously (11). The plasmid subclone, pBLV913, consists of the 5′ *Eco*RI fragment of clone 9 and the 3′ *Eco*RI fragment of clone 13 with 400 bp of 5′ cellular flanking sequence and 3 bp of 3′ flanking sequence cloned into *Kpn*I-modified *Pvu*II sites of pUC9 (9).

Microinjection of BESP cells. Microinjection was performed by a modified version of the technique of Diacumakos (13) as previously described (3). BESP cells were seeded at 10^6 cells per 60-mm dish and grown overnight prior to injection. Lambda DNAs were purified on cesium gradients and quantitated spectrophotometrically. Ten femtoliters of a 100-μg/ml solution of each BLV clone DNA was injected directly into the nuclei of 200 cells. This procedure was repeated in seven separate experiments with multiple preparations of the individual clones.

BLV expression in microinjected cells. After microinjection of BESP cells, syncytia (cells containing at least five nuclei) were observed and counted with a phase-contrast microscope. BLV expression was monitored by indirect immunofluorescence assay (IFA) (4) with polyclonal rabbit sera against BLV p24 and monoclonal antibody against gp51 (supplied by Daniel Portetelle, Department of Microbiology, Gembloux, Belgium) in situ each day for the first 12 days. Controls included normal rabbit sera and irrelevant, isotype monoclonal antibodies. Subsequently, IFAs were performed on passaged cells, and samples of positive cultures were prepared for examination by electron microscopy as previously described (4) and viewed in a Hitachi H-7000 electron microscope at 75 kV.

Reverse transcriptase (RT) assays were performed as described by Gonda et al. (23) with protocols of Hoffman et al. (28). Virus was pelleted from clarified cell supernatants, and RT activity was measured with poly(rA)-oligo$(dT)_{12-18}$ template-primer, 20 mM Mg^{2+}, appropriate deoxynucleoside triphosphates (dNTPs), and [^{3}H]TTP. Assays were performed in triplicate, and results were adjusted to counts per minute of incorporated ^{3}H per milliliter of virus-containing culture medium to permit comparisons.

Experimental animals. The procedure used for experimental infection of the nine sheep used in this study has been described previously (31) and is summarized in Table 1. Briefly, two Suffolk sheep (no. 41 and 84) were inoculated intraperitoneally at 3 months of age with 10^7 clone-infected BESP cells (BESP-913), established by microinjection with pBLV913. These sheep were 57 months postinfection (p.i.) by the end of the present study. At 15 months p.i., PBMC from these two sheep were transferred intravenously to four 3-month-old Suffolk sheep. Sheep L1 and L2 received 10^8 PBMC from sheep 41, and sheep L3 and L4 received 10^8 PBMC from sheep 84. These sheep were 42 months p.i. by the end of the present study. For comparison with infection with a naturally occurring BLV isolate, two Rambouillet sheep (no. 3 and 5), which had been inoculated intraperitoneally at 3 months of age with 10^7 PBMC from a BLV-seropositive cow with PL, were included. These sheep were 70 months p.i. by the end of the present study. One Suffolk sheep (no. 94) was inoculated intraperitoneally with 3×10^7 uninfected, primary bovine leukocyte adherent cells to serve as a negative control. In addition, five normal Rambouillet sheep, between 2 and 5 years of age, provided average control values for lymphocyte enumeration.

Cell isolation, serology, and hematology. Venous blood, with EDTA as anticoagulant, was collected, and total and differential leukocyte counts were determined manually. PBMC were isolated by density gradient centrifugation on Ficoll-Hypaque (Histopaque 1077; Sigma Chemical Co., St. Louis, Mo.). Anti-BLV antibody titers in serum were determined with a competitive enzyme-linked immunosorbent assay as previously described (43) with reagents kindly supplied by Daniel Portetelle.

Flow cytometric analysis. For analysis of lymphocyte subsets, 10^6 PBMC were suspended in 35 μl of the appropriate dilution of monoclonal antibody. Cells were incubated at 4°C for 20 min, washed twice in 0.5 ml of phosphate-buffered saline (PBS) with 1% bovine serum albumin and 0.1% NaN_3, and resuspended in 35 μl of a 1:50 dilution of affinity-purified goat anti-mouse IgG conjugated to fluorescein isothiocyanate (IgG-FITC) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). After a repeat of the incubation and wash, cells were analyzed by flow cytometry. Controls for each PBMC preparation included irrelevant isotype antibodies and goat anti-mouse IgG-FITC alone. Monoclonal antibodies used to identify cell surface antigens of sheep lymphocytes included SBU II for MHC II (45), SBU-T1 for $CD5^+$ T cells, and SBU-T6 for CD1c, which marks B cells in sheep (38). Ovine sIg was detected directly with a 1:50 dilution of affinity-purified goat anti-sheep IgG-FITC (heavy and light chains) (Kirkegaard & Perry Laboratories). Additional analyses of sIg were performed with affinity-purified goat anti-sheep IgG-FITC (γ chain specific) (Kirkegaard & Perry Laboratories) and monoclonal antibodies specific for bovine Ig subtypes with documented cross-reactivity to ovine gamma globulins (27) supplied by Klaus Nielsen (Agriculture Canada, Nepean, Ontario, Canada); IgG1 (M23), IgG2 (M37), IgM (M33), and IgA (M67). Dual labeling of cells for sIg and CD5 was performed by consecutive applications of SBU-T1, goat anti-mouse IgG-phycoerythrin (Kirkegaard & Perry Laboratories), and goat anti-sheep IgG-FITC.

Cell surface label was analyzed with a Coulter EPICS Profile II flow cytometer equipped with a 488-nm argon laser. Ten thousand lymphocytes, defined by forward angle versus side light scatter, were analyzed. Analysis regions were defined to include less than 2% of preparations stained with irrelevant primary antibody.

Immunofluorescence assay. For detection of virus expression, PBMC were cultured at a concentration of 2.5×10^6 per ml of RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), L-glutamine (4 mM), 1% nonessential amino acids (GIBCO-50×), 2% essential amino acids (GIBCO-100×), β-mercaptoethanol (20 μM), 20% fetal calf serum (HyClone

Laboratories, Inc., Logan, Utah), and 50 μg of phytohemagglutinin M (PHA-M; Difco Laboratories, Detroit, Mich.) per ml for 12 h. Cells were washed and resuspended in PBS at 2×10^7 cells per ml. Five microliters was deposited in 4-mm wells on glass slides (Cel-Line Associates, Inc., Newfield, N.J.), dried, and fixed for 5 min in cold acetone-methanol (1:1). IFA detection of BLV expression was performed as previously described (50) with polyclonal rabbit sera against BLV. Control preparations included freshly isolated PBMC, normal rabbit serum, and monoclonal antibodies for BLV p24 and gp51. A minimum of 200 cells were counted by fluorescence microscopy for determination of the percent positive cells in each preparation.

Lymphocyte proliferation. The procedures of Cockerell et al. (7) were used for the lymphocyte proliferation assay. Briefly, 10^5 PBMC were cultured per well in a 96-well plate. Cultures were supplemented with either no mitogen, PHA-M (50 μg/ml), or formalinized *Staphylococcus aureus* Cowan (20 bacteria per lymphocyte). Cultures were incubated for 3 days prior to addition of 0.5 μCi of [*methyl*-^{3}H]thymidine per well for an additional 18 h. The total contents of each well were then collected onto glass fiber filters and counted by liquid scintillation for determination of thymidine incorporation. All assays were performed in triplicate.

Nucleic acid isolation and analysis. The procedures for DNA and RNA isolation and Southern and Northern (RNA) blotting were performed as previously described (8, 50). Autoradiographs were digitized on a flatbed scanner (HP Scanjet; Hewlett-Packard, Palo Alto, Calif.) for densitometric analysis (Scan Analysis; Biosoft, Ferguson, Mo.).

The polymerase chain reaction (PCR) was used to amplify BLV *pol* and *tax* sequences. The sequences and positions in the BLV genome (52) of the primers used were as follows: RTa, 5′-GCATGACCTA CGAGCTACAA-3′ (nucleotides [nt] 2469 to 2488); RTb, 5′-GCGTAAAGGA TATCGTCCAT-3′ (nt 2819 to 2800); TAXa, 5′-GCCATACGTT ATCTCTCCA C-3′ (nt 7795 to 7814); and TAXb, 5′-GGTTAGCGTA GGGTCATGAA-3′ (nt 8079 to 8060). Purified PBMC DNA or FLK-BLV DNA (1 μg) was subjected to 30 amplification cycles in a 50-μl reaction volume containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 8 mM $MgCl_2$, 1 mg of gelatin per ml, 1% glycerol, 200 μM dNTPs, 0.2 μM primers, and 2.5 U of *Taq* polymerase (Boehringer Mannheim Corp., Indianapolis, Ind.). Each cycle consisted of 1 min at 96°C, 1 min at 63°C, and 20 s at 72°C. A total of 20 μl of product (2 μl from FLK-BLV DNA product) was separated by electrophoresis in 3% Nusieve–1% GTG agarose and transferred to a nylon membrane. Blots were hybridized with ^{32}P-labeled probes with the primer sequences excluded.

RESULTS

Molecular clones of BLV. Southern blots of restriction digestions of the individual BLV proviral clones from the FLK-BLV genome are presented in Fig. 1. *Sst*I digestion of the four BLV proviral isolates produced the same two major hybridizing bands at approximately 7.1 and 1.2 kb in length. Minor hybridizing bands represent virus-host junction fragments, some of which are linked to the lambda arm. Alternatively, digestion of the four BLV proviral isolates with *Eco*RI produced two hybridizing bands of different molecular weights for each isolate, showing that they are unique.

BLV clones in vitro. The biological activity of the BLV proviral clones following microinjection of BESP cells is summarized in Table 2. Clones 9 and pBLV913 consistently induced syncytia within 48 h, clone 8 induced syncytia within 3 to 4 days, and clone 13 did so within 7 to 8 days, while clone 1 did not induce syncytia. Approximately 10% of microinjected cells produced syncytia. The BLV-induced syncytia were large cells with 5 to 20 adjacent nuclei surrounded by abundant cytoplasm (Fig. 2A). BLV expression was confirmed by IFA on cells in situ. Polyclonal rabbit sera specific for BLV p24 and monoclonal antibodies against BLV gp51 reacted with syncytia and morphologically normal cells 2 days after injection with BLV clone 9 and pBLV913. BESP cells microinjected with clone 8 or 13 first expressed BLV 3 or 8 days postinjection, respectively, concurrent with syncytium formation. In virus-expressing cells, p24 IFA was intense and localized to the cytoplasm (Fig. 2B); gp51 IFA was less intense and localized on the membrane (not shown). No BLV antigens were detected in uninjected BESP cells or in BESP cells injected with clone 1.

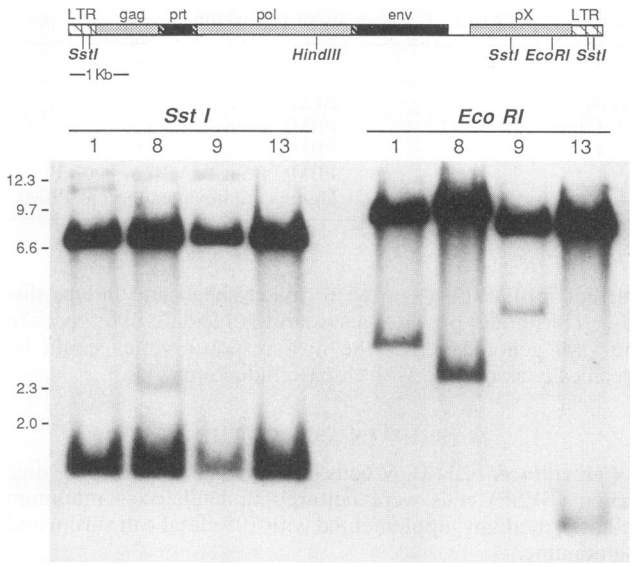

FIG. 1. Partial restriction map of the BLV genome and Southern analysis of the FLK-BLV proviral clones. Approximately 1 μg of each lambda isolate was digested with the indicated restriction endonuclease. Hybridization was carried out with a representative probe (specific activity = 10^8 cpm/μg).

RT assays of cell-free supernatants from microinjected cells showed low levels of RT activity from clone 9 and pBLV913 by passage 4, from clone 8 by passage 6, and from clone 13 by passage 8. RT activity increased with successive passages of all positive cultures; values for RT activity from BESP-913 are presented in Fig. 3. No RT activity was detected in supernatants of clone 1-injected cells. Cells were examined by electron microscopy after eight passages. Budding virus particles were

TABLE 2. In vitro activity of BLV clones[a]

Clone no.	Syncytia	p24	RT	EM[b]	Transfer of infection
1	− (d12)	− (d12)	− (P8)	− (P8)	− (P12)
8	+ (d3)	+ (d3)	+ (P6)	ND[c]	+ (P9)
9	+ (d2)	+ (d2)	+ (P4)	+ (P8)	+ (P9)
13	+ (d8)	+ (d8)	+ (P8)	ND	+ (P12)
pBLV913	+ (d2)	+ (d2)	+ (P4)	+ (P8)	+ (P9)

[a] −, negative; +, positive; d or P, day or passage of earliest detection.
[b] EM, virus particles detected by electron microscopy.
[c] ND, not done.

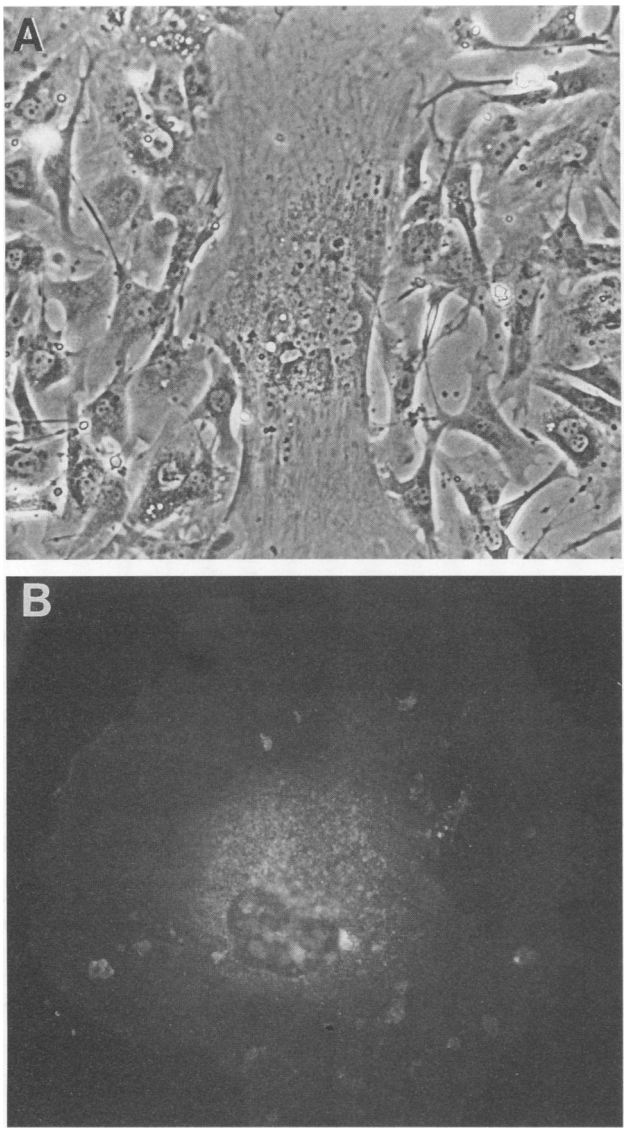

FIG. 2. BESP cells microinjected with clone 913. (A) Phase-contrast micrograph of syncytia associated with BLV expression; (B) cytoplasmic BLV p24 antigen detected by immunofluorescence assay.

observed in 1 of 12 thin sections of cells injected with clones 9 and pBLV913 examined (not shown). No particles were detected in clone 1-injected cells. Budding and extracellular virus were easily observed with FLK-BLV cells. BLV infection was transferred to naive BESP cells with cell-free supernatants from all clone-injected cells except those injected with clone 1. These cells developed syncytia and were positive for BLV antigen expression and RT activity in subsequent cultures.

pBLV913 in BESP cells and sheep. BESP-913 cells were expanded continuously in culture for 12 months prior to inoculation of sheep 41 and 84. Cells were 30% positive for BLV antigen by IFA and produced 1/10 of the levels of RT activity observed in FLK-BLV cell supernatants. Southern analysis of *Sst*I digests of genomic DNA from these cells indicated that the integrated proviral load was comparable to that observed in FLK-BLV cells (four copies per cell) (Fig. 4A). *Hin*dIII digestion produced two to four visible bands and an array of heterogeneous fragment sizes. This indicated the

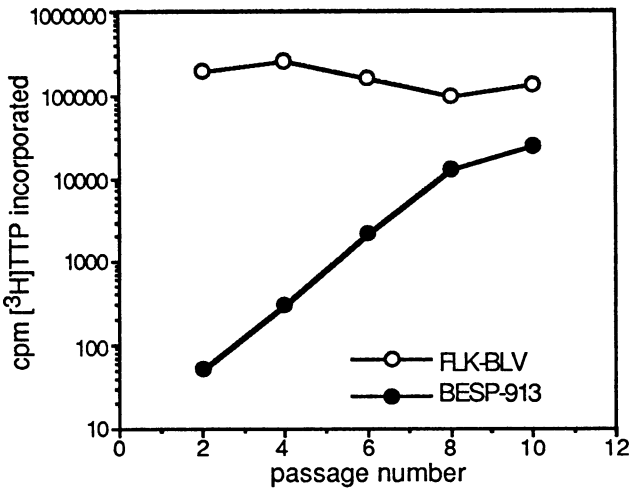

FIG. 3. RT activity of cell supernatants from BESP-913 cells and positive control FLK-BLV cells. Passage number refers to microinjected cells only; the corresponding FLK supernatant was collected at each time interval.

appearance of a clonal subpopulation of cells among cells with polyclonally integrated provirus (Fig. 4B). Northern blots of poly(A)$^+$ RNA from BESP-913 cells demonstrated levels of BLV message comparable to that in FLK-BLV cells (not shown).

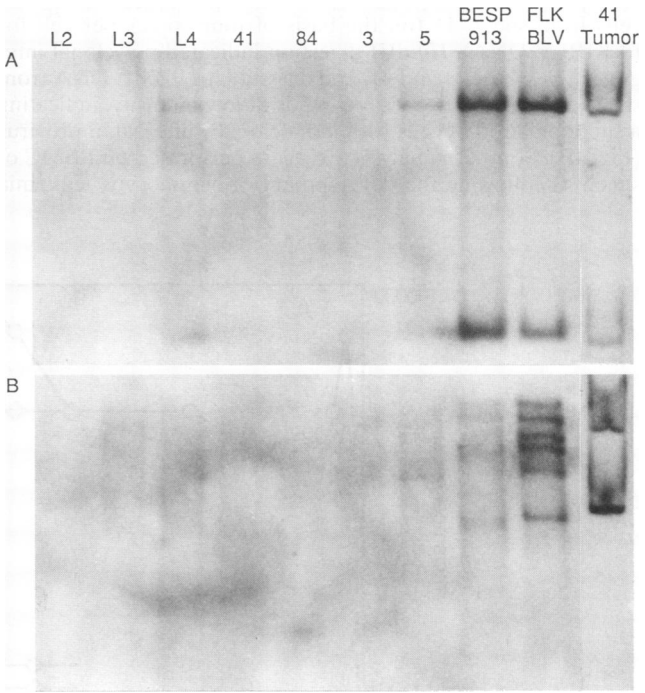

FIG. 4. Southern analysis of PBMC DNA of infected sheep, BESP-913 cells, FLK-BLV cells, and leukemic cells of sheep 41. (A) Determination of proviral load with *Sst*I digestion; (B) digestion at the internal *Hin*dIII site which yields two bands from each clonally integrated BLV provirus. Sheep 41 and 84 were 53 months p.i.; L2 to L4 were 38 months p.i.; no. 3 and 5 were 66 months p.i. BESP-913 DNA was prepared at the time of infection of sheep 41 and 84. Leukemic cells of sheep 41 were taken at 57 months p.i.

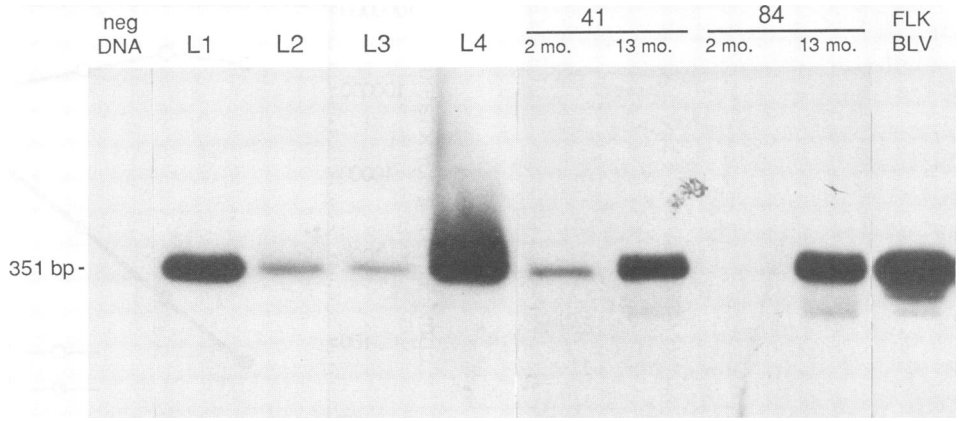

FIG. 5. Analysis of PCR products amplified from PBMC DNA of clone-infected sheep early in infection. The 351-bp *pol* product was first detected in PBMC of sheep inoculated with BESP-913 cells (no. 41 and 84) at 2 and 13 months p.i., respectively. The *pol* product was detected at 2 months p.i. in PBMC of sheep transfused with PBMC from sheep 41 and 84. A total of 20 μl of PCR product of sheep PBMC DNA and 2 μl of product of FLK-BLV DNA were subjected to electrophoresis. Uninfected sheep PBMC DNA served as the negative control (neg).

Southern analysis of *Sst*I digests of PBMC DNA from infected sheep yielded detectable signal only from clone-infected sheep L4 and 41 at 38 and 53 months p.i., respectively, and sheep 5, which received BLV^+ bovine PBMC, at 66 months p.i. (Fig. 4A). Provirus on Southern blot was first detected in these animals at 38, 37, and 54 months p.i., respectively. Comparison of the intensity of bands on Southern blots by densitometry indicated approximately one proviral copy per cell from sheep L4 and 5 and one copy for every five cells from sheep 41 (on the basis of four copies per cell for FLK-BLV DNA). *Hin*dIII digestion indicated polyclonal integration in sheep L4 and 41, and digestion of PBMC DNA from sheep 5 produced two visible bands of low intensity, indicating a subpopulation of cells with monoclonally integrated provirus (Fig. 4B). Southern analysis was again performed on PBMC of sheep 41 following the development of lymphocytic leukemia at 57 months p.i. and indicated that the leukemic cells harbored a single, full-length BLV provirus (Fig. 4).

Provirus was readily detected in all infected sheep after amplification of 1-μg quantities of purified PBMC DNA by PCR with either *pol*- or *tax*-specific primers (Fig. 5) (*tax* products not shown). Provirus was first detected in PBMC of sheep 41 and 84, which received BESP-913 cells, at 2 and 13 months p.i., respectively. Sheep L1, L2, L3, and L4, which received PBMC from sheep 41 or 84, all had detectable provirus at 2 months p.i.

Serology. Sheep 41 and 84 seroconverted to gp51 at 3 weeks p.i. Following early peaks, antibody titers stabilized at approximately $1:10^4$ by 3 years p.i. (Fig. 6A). At that time, there was approximately a 10-fold increase in the antibody titer of sheep 41. Two of the sheep which received PBMC, either from no. 41 or from no. 84 (one in each group) seroconverted at 5 and 4

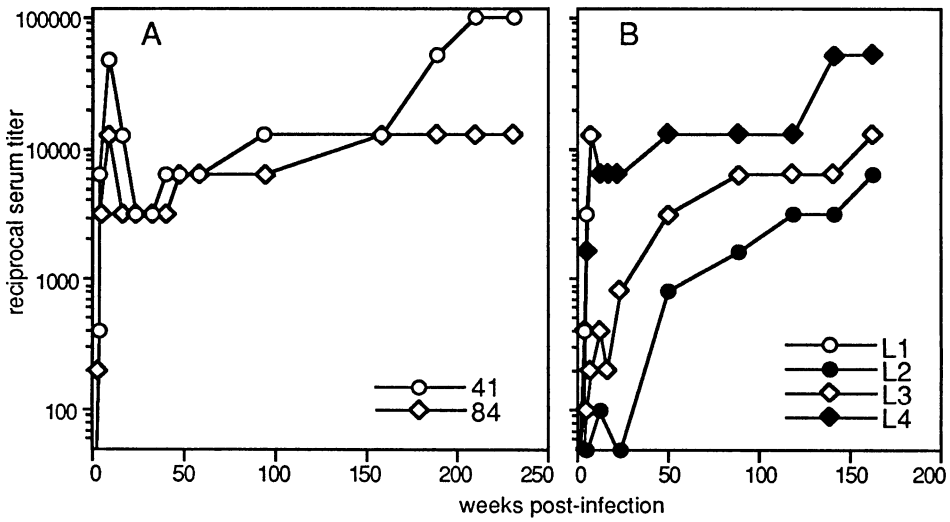

FIG. 6. Anti-BLV gp51 serum titers of clone-infected sheep. (A) Sheep 41 and 84 inoculated with BESP-913 cells; (B) sheep L1 to L4 transfused with PBMC from no. 41 (L1 and L2) or no. 84 (L3 and L4). Sheep L1 died of unrelated causes at 2 months p.i. Titers were based on >20% competition with serum antibody with peroxidase-conjugated anti-BLV gp51 monoclonal antibody.

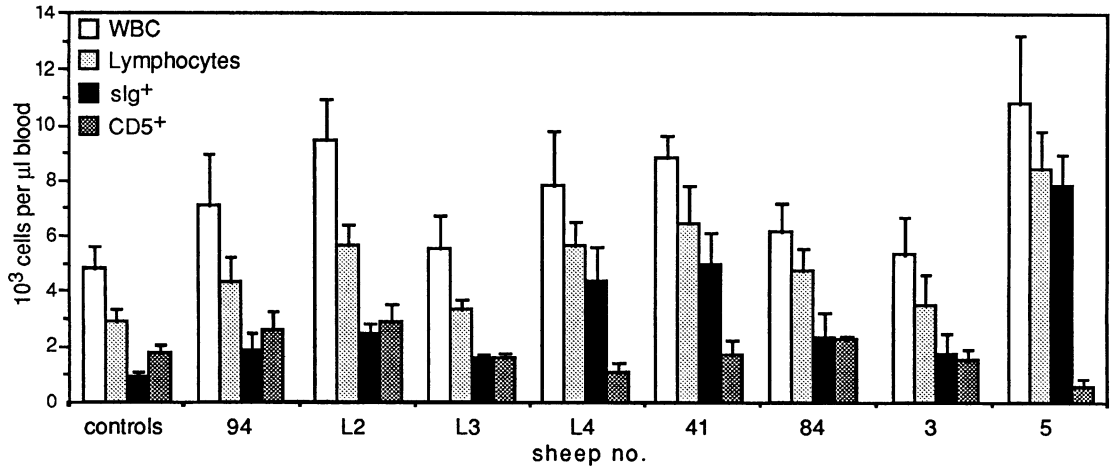

FIG. 7. Enumeration of leukocytes (WBC), lymphocytes, and sIg^+ and $CD5^+$ PBMC from control and infected sheep. Data from individual animals represent mean and standard error of the mean of values obtained from four separate bleed dates over a 6-week period ending when sheep were 38 months p.i. (L2 to L4), 53 months p.i. (no. 41 and 84), or 66 months p.i. (no. 3 and 5). Control data represent values from single bleeds of five age-matched animals.

weeks p.i. (L2 and L3, respectively) (Fig. 6B). Antibody titers in these sheep increased gradually to approximately $1:10^4$. Sheep L1 and L4 seroconverted at 3 weeks p.i. with relatively high titers. Sheep L1 died from unrelated causes at 2 months p.i. Following an early peak, the antibody titer of sheep L4 stabilized at approximately $1:10^4$ and then increased fivefold at 2.5 years p.i. The sham-inoculated sheep, no. 94, had no detectable anti-BLV antibodies. Sheep 3 and 5 seroconverted to BLV gp51 at 4 weeks p.i. and developed serum antibody titers of 1:51,200 and 1:102,400, respectively, by 50 months p.i. (not shown).

Hematology. Values for total leukocytes, lymphocytes, and sIg^+ and $CD5^+$ lymphocyte subpopulations are shown in Fig. 7. Clone-infected sheep 41 and 84 developed inverted or equivalent B/T-lymphocyte numbers at 48 and 53 months p.i., respectively. Similar changes developed in sheep L3, L4, 3, and 5 at 38, 33, 60, and 16 months p.i., respectively. Sheep L2 did not exhibit overt hematological abnormalities. Of the affected animals, L4, sheep 41 and 5 developed B lymphocytosis. Flow cytometric analysis demonstrated an increase in expression of sIg in the expanded B-cell populations of these sheep (Fig. 8A). High-intensity fluorescence was also observed with monoclonal antibodies for IgM, MHC II, and CD1c, but not for ovine IgG γ chain or Ig subtypes IgG1, IgG2, or IgA (not shown). The presence of IgD was not determined. The onset of leukemia in sheep 41 was evidenced by a total leukocyte count of $1.27 \times 10^6/\mu l$ consisting of more than 99% lymphoblasts which stained intensely for sIg, IgM, CD1c, and MHC II (Fig. 8B). The antibody used to detect ovine CD5, SBU-T1, labeled approximately 20% of sIg^+ cells with low intensity. This population was excluded from $CD5^+$ totals. Leukemic cells from sheep 41 were CD5 negative.

Lymphocyte proliferation. Spontaneous proliferation was increased in cultures of PBMC from all BLV-infected sheep except sheep 3, and a direct correlation existed between spontaneous blastogenesis and *S. aureus* Cowan-induced B-cell blastogenesis (mean stimulation index = 5.2 ± 0.85) (Fig. 9). The PHA-M response was low in six of seven BLV-infected sheep compared with the control sheep.

BLV expression in cultured PBMC. Short-term cultured PBMC from all BLV-infected sheep included cells which expressed BLV as determined by IFA (Fig. 10). At 53 months p.i., 5 and 1% of PBMC from sheep 41 and 84, respectively, were BLV^+. At 38 months p.i., sheep L2, L3, and L4 had 0.1, 1.5, and 16% BLV^+ PBMC, respectively. BLV^+ PBMC accounted for 6 and 79% of PBMC from sheep 3 and 5, respectively. Cocultures of PBMC from all BLV-infected sheep with uninfected BESP cells yielded syncytia and IFA-detectable BLV antigens in the target cells (not shown). Syncytium induction correlated with numbers of IFA^+ PBMC. Leukemic cells from sheep 41 were BLV^- after in vitro cultivation, and cocultures with BESP cells did not result in syncytium induction or BLV expression. $Poly(A)^+$ RNAs, isolated from both fresh and cultured leukemic cells from sheep 41, were negative for BLV-specific mRNAs when probed with the 1.2-kb *Sac*I fragment of pBLV913 (not shown). The presence of intact mRNAs was confirmed with a bovine actin probe.

DISCUSSION

Four BLV clones derived from FLK-BLV and one plasmid subclone, pBLV913, were assayed for infectivity. Clone 9 and pBLV913 demonstrated the greatest replicative competence, clones 8 and 13 were intermediate in their ability, and clone 1 was defective. The onset of cytopathic effect and virus expression were consistent between repeated experiments with new DNA preparations, indicating a direct relationship to the sequences of the individual clones. The functional characteristics of the LTRs of the clones, evaluated previously, had no differences in promotional capacity (11), and restriction analysis has shown that each clone represents a full-length BLV provirus. This excludes roles for substantial deletions and/or deficits in the promoter regions in the variation of expression. Sequence analysis of the structural and regulatory genes will be necessary to further delineate the mechanisms responsible.

A manifestation of BLV-induced disease is the abnormal, persistent expansion of the peripheral B-lymphocyte population (32, 34, 41) accompanied by a high antiviral serum titer (36). This condition predisposes cattle to the development of leukemia (18, 32) and frequently precedes leukemia in experimentally infected sheep (14, 16). Infection with BESP-913 cells resulted in B lymphocytosis in one of two sheep after a prolonged incubation period, and culminated in B-cell leuke-

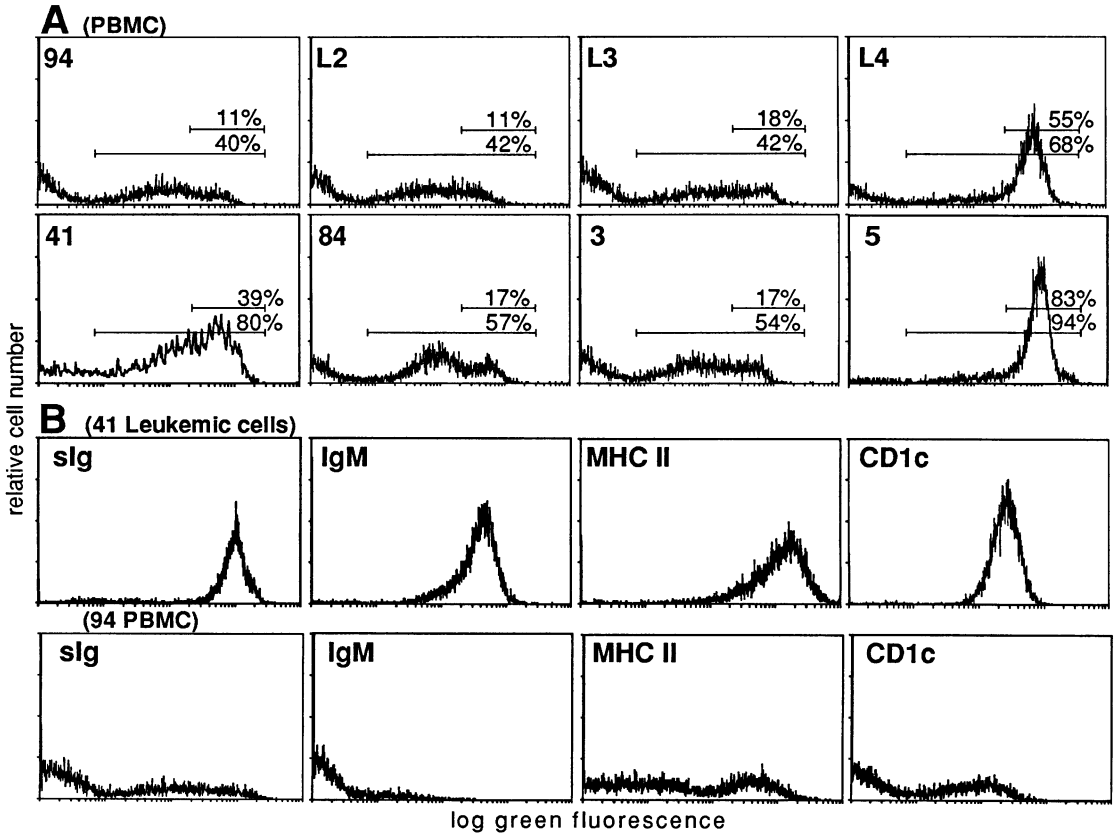

FIG. 8. Flow cytometric analysis of surface label on sheep PBMC and leukemic cells. (A) Detection of sIg on PBMC; two analysis regions include (i) all sIg^+ cells (less than 2% of negative control labeled cells) and (ii) a subpopulation of intensely staining cells. The percent positive cells within each region are indicated. Sheep were 38 months p.i. (L2 to L4), 53 months p.i. (no. 41 and 84), or 66 months p.i. (no. 3 and 5). These results were representative of four separate experiments over a 6-week period. (B) Analysis of expression of sIg, IgM, MHC II, and CD1c by leukemic cells from sheep 41 at 57 months p.i. compared with analysis of PBMC from control sheep 94.

mia. Transfer of lymphocytes from these sheep early in infection (15 months) to four additional sheep resulted in B lymphocytosis in one of three surviving sheep. Overall, an altered B/T-lymphocyte ratio was observed in four of five clone-infected sheep. The results were comparable to the conditions induced in cattle and sheep with natural BLV isolates. The B cells from sheep with B lymphocytosis, and, subsequently, leukemic cells from one clone-infected sheep, were characterized by a uniform upregulation of surface IgM and MHC II, indicating an activated state. B cells of this type are capable of interaction with class II restricted, antigen-specific helper T cells (6). This phenotype is comparable to that of most BLV-associated tumor cells and is in support of a differentiation-linked mechanism of leukemogenesis (26).

Lymphocyte proliferation assays demonstrated the spontaneous growth of lymphocytes from affected sheep upon in vitro cultivation. This phenomenon is coincident with BLV expression and common to PBMC of animals with PL. The levels of spontaneous thymidine uptake were proportional to uptake in cultures stimulated with the T-cell-independent, B-cell mitogen, *S. aureus* Cowan. This suggests that it is the B-lymphocyte population which undergoes division upon in vitro cultivation. Response to the T-cell mitogen, PHA, was decreased in all but one BLV-infected sheep. Reduced PHA response of PBMC from sheep and cattle with PL has been previously ascribed to reduced T-cell numbers (16, 53). The reduced response to PHA of PBMC from animals with normal T-cell numbers, however, indicates a deficit in T-cell response to PHA.

Aberrations in B-cell numbers correlated with viral load as determined by Southern analysis. Likewise, virus expression in vitro was greatest in PBMC of these sheep. Leukemic cells from sheep 41 did not express virus upon in vitro cultivation, and Northern analysis of mRNA from both fresh and cultured leukemic cells showed no detectable viral message. The inability to induce BLV expression in vitro from an apparently complete provirus has been described for some BLV-induced tumors (54) and indicates that virus expression, including *tax*, is not necessary for maintenance of the transformed state. Cloned, full-length proviruses from such tumors have been found to be competent when transfected in mammalian cell lines (54). These results indicate a cellular mechanism in the repression of viral gene expression and in the maintenance of the transformed state.

Southern analysis of PBMC DNA from sheep 5, infected with noncloned BLV, indicated the presence of clonal, BLV^+ cells, and most of the PBMC from this sheep expressed virus in vitro. BLV clone-infected sheep, 41 and L4, were comparable to sheep 5 in B-cell number and character, total proviral load, and virus-positive cells after cultivation in vitro. However, the status of BLV integration in the PBMC of these sheep remained polyclonal (prior to onset of leukemia in sheep 41), and virus-expressing cells were 5 and 15 times less. The

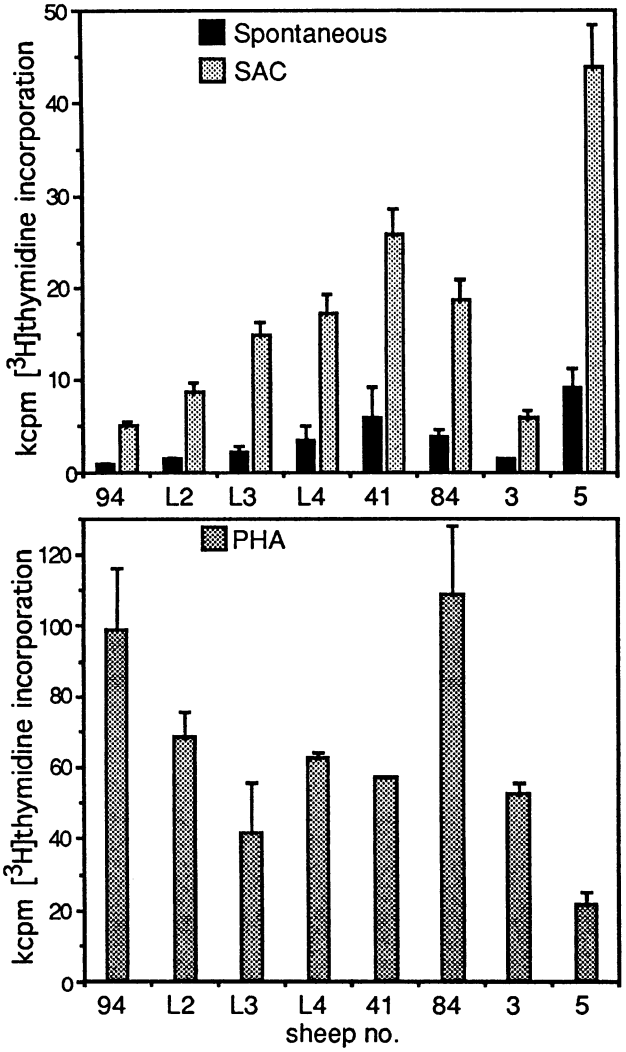

FIG. 9. Spontaneous, *S. aureus* Cowan, and PHA-induced blastogenesis of PBMC from control and infected sheep. The average stimulation index for *S. aureus* Cowan was 5.2 ± 0.85. Data were from a single bleed when sheep were 38 months p.i. (L2 to L4), 53 months p.i. (no. 41 and 84), or 66 months p.i. (no. 3 and 5). These results were representative of four separate experiments over a 6-week period.

development of leukemia in sheep 41, while sheep 5 and L4 remained essentially unchanged, suggests that additional random events are responsible for the generation of the transformed phenotype.

Experimental infection of sheep with a single molecular clone of BLV yielded disparate host responses. A strong initial serum antibody response was prognostic for development of hematological abnormalities and high proviral load. Total lymphocyte counts at the time of seroconversion were greater in sheep which progressed to B lymphocytosis and lymphoma, as has been shown previously in experimentally infected sheep (15, 56). These results suggest an important role for host immune response in the progression of virus replication and pathogenesis (16, 35, 44) and support previous studies which identify genetic susceptibility for BLV pathogenesis in cattle (19, 37, 42). Analyses of BLV isolates from diverse geographical origins and molecular clones recovered from transfected

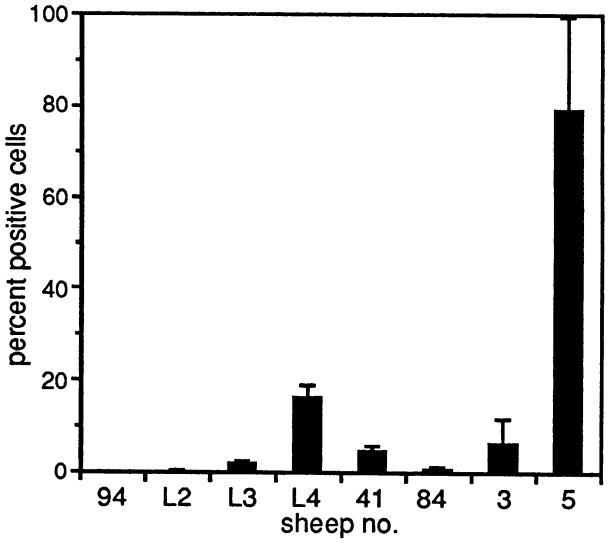

FIG. 10. Percent of BLV$^+$ cells after 12 h of in vitro cultivation. Data represent the mean and standard error of the mean of values obtained from three separate bleed dates over a 6-week period (see legend to Fig. 7).

animals have indicated a stable genome (39, 49, 59). The possibility remains that pathogenic variants of the original pBLV913 clone may have arisen in vivo. The proviruses from the clone-infected sheep and BESP-913 inoculum are now subject to sequence analysis to determine the presence of such variants.

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REFERENCES

1. **Alexandersen, S., S. Carpenter, J. Christensen, T. Storgaard, B. Viuff, Y. Wannemuehler, J. Belousov, and J. A. Roth.** 1993. Identification of alternatively spliced mRNAs encoding potential new regulatory proteins in cattle infected with bovine leukemia virus. J. Virol. **67**:39–52.
2. **Baliga, V., and J. F. Ferrer.** 1977. Expression of bovine leukemia virus and its internal antigen in blood lymphocytes. Proc. Soc. Exp. Biol. Med. **156**:388–391.
3. **Boyd, A. L.** 1985. Expression of cloned genes microinjected into cultured mouse and human cells. Gene Anal. Tech. **2**:1–9.
4. **Boyd, A. L., T. G. Wood, A. Buckley, P. J. Fischinger, R. V. Gilden, and M. A. Gonda.** 1988. Microinjection and expression of an infectious proviral clone and subgenomic envelope construct of a human immunodeficiency virus. AIDS Res. Human Retroviruses **4**:31–41.
5. **Brenner, J., M. Van-Haam, D. Savir, and Z. Trainin.** 1989. The implications of BLV infection in the productivity, reproductive capacity and survival rate of a dairy cow. Vet. Immunol. Immunopathol. **22**:299–305.
6. **Cambier, J. C., and J. T. Ransom.** 1987. Molecular mechanisms of transmembrane signaling in B lymphocytes. Annu. Rev. Immunol. **5**:175–199.
7. **Cockerell, G. L., E. A. Hoover, A. F. LoBuglio, and D. S. Yohn.** 1975. Phytomitogen- and antigen-induced blast transformation of feline lymphocytes. Am. J. Vet. Res. **36**:1489–1494.

8. **Cockerell, G. L., and J. Rovnak.** 1988. The correlation between the direct and indirect detection of bovine leukemia virus infection in cattle. Leuk. Res. **12:**465–469.
9. **Derse, D.** 1987. Bovine leukemia virus transcription is controlled by a virus-encoded *trans*-acting factor and by *cis*-acting response elements. J. Virol. **61:**2462–2471.
10. **Derse, D.** 1988. *trans*-Acting regulation of bovine leukemia virus mRNA processing. J. Virol. **62:**1115–1119.
11. **Derse, D., S. J. Caradonna, and J. W. Casey.** 1985. Bovine leukemia virus long terminal repeat: a cell type-specific promoter. Science **227:**317–320.
12. **Deshayes, L., D. Levy, A. L. Parodi, and J. P. Levy.** 1980. Spontaneous immune response of bovine leukemia virus-infected cattle against five different viral proteins. Int. J. Cancer **25:**503–508.
13. **Diacumakos, E. G.** 1973. Methods for micromanipulation of human somatic cells in culture. Methods Cell Biol. **8:**287–312.
14. **Dimmock, C. K., R. J. Rogers, Y. S. Chung, A. R. Mackenzie, and P. D. Waugh.** 1986. Differences in the lymphoproliferative response of cattle and sheep to bovine leucosis virus infection. Vet. Immunol. Immunopathol. **11:**325–331.
15. **Dimmock, C. K., W. H. Ward, and K. F. Trueman.** 1989. Lymphocyte subpopulations in sheep during the early stage of experimental infection with bovine leukaemia virus. Immunol. Cell Biol. **67:**141–145.
16. **Djilali, S., and A. L. Parodi.** 1989. The BLV-induced leukemia-lymphosarcoma complex in sheep. Vet. Immunol. Immunopathol. **22:**233–244.
17. **Djilali, S., A. L. Parodi, D. Levy, and G. L. Cockerell.** 1987. Development of leukemia and lymphosarcoma induced by bovine leukemia virus in sheep: a hematopathological study. Leukemia **1:**777–781.
18. **Ferrer, J. F.** 1980. Bovine lymphosarcoma. Adv. Vet. Sci. Comp. Med. **24:**1–68.
19. **Ferrer, J. F., R. R. Marshak, D. A. Abt, and S. J. Kenyon.** 1979. Relationship between lymphosarcoma and persistent lymphocytosis in cattle: a review. J. Am. Vet. Med. Assoc. **175:**705–708.
20. **Fossum, C., A. Burny, D. Portetelle, M. Mammerickx, and B. Morein.** 1988. Detection of B and T cells with lectins or antibodies in healthy or bovine leukemia virus-infected cattle. Vet. Immunol. Immunopathol. **18:**269–278.
21. **Franchini, G., F. Wong-Stall, and R. C. Gallo.** 1984. Human T-cell leukemia virus (HTLV-1) transcripts in fresh and cultured cells of patients with adult T-cell leukemia. Proc. Natl. Acad. Sci. USA **81:**6207–6211.
22. **Ghysdael, J., C. Bruck, R. Kettmann, and A. Burny.** 1984. Bovine leukemia virus. Curr. Top. Microbiol. Immunol. **112:**1–19.
23. **Gonda, M. A., M. J. Braun, S. G. Carter, T. A. Kost, J. W. Bess, L. O. Arthur, and M. J. Van der Maaten.** 1987. Characterization and molecular cloning of a bovine lentivirus related to human immunodeficiency virus. Nature (London) **330:**388–391.
24. **Haas, L., T. Divers, and J. W. Casey.** 1992. Bovine leukemia virus gene expression in vivo. J. Virol. **66:**6223–6225.
25. **Heeny, J. L., P. J. S. Valli, R. M. Jacobs, and V. E. O. Valli.** 1992. Evidence for bovine leukemia virus infection of peripheral blood monocytes and limited antigen expression in bovine lymphoid tissue. Lab. Invest. **66:**608–617.
26. **Heeny, J. L., and V. E. O. Valli.** 1990. Transformed phenotype of enzootic bovine lymphoma reflects differentiation-linked leukemogenesis. Lab. Invest. **62:**339–346.
27. **Henning, D., and K. Nielsen.** 1992. Cross-reactivity of monoclonal antibodies to bovine immunoglobulins with immunoglobulins of other species. Vet. Immunol. Immunopathol. **34:**235–243.
28. **Hoffman, A. D., B. Banapour, and J. A. Levy.** 1985. Characterization of the AIDS-associated retrovirus reverse transcriptase and optimal conditions for its detection in virions. Virology **147:**326–335.
29. **Ishino, S., I. Matsuda, H. Yamamoto, T. Yoshino, H. Sentsui, Y. Mizuno, and Y. Kono.** 1989. Pathological findings of two types of lymphoid malignancy in sheep inoculated with bovine leukemia virus. Jpn. J. Vet. Sci. **51:**749–756.
30. **Itohara, S., and K. Sekikawa.** 1987. Molecular cloning of infectious proviral genomes of bovine leukemia virus. Virology **159:** 158–160.
31. **Jensen, W. A., J. Rovnak, and G. L. Cockerell.** 1991. In vivo transcription of the bovine leukemia virus *tax/rex* region in normal and neoplastic lymphocytes of cattle and sheep. J. Virol. **65:**2484–2490.
32. **Kettmann, R., Y. Cleuter, M. Mammerickx, M. Meunier-Rotival, G. Bernardi, A. Burny, and H. Chantreene.** 1980. Genomic integration of bovine leukemia virus: comparison of persistent lymphocytosis with lymph node tumor form of enzootic bovine leukosis. Proc. Natl. Acad. Sci. USA **77:**2577–2581.
33. **Kettmann, R., G. Marbaix, Y. Cleuter, D. Portetelle, M. Mammerickx, and A. Burny.** 1980. Genomic integration of bovine leukemia provirus and lack of viral RNA expression in the target cells of cattle with different responses to BLV infection. Leuk. Res. **4:**509–519.
34. **Kumar, S. P., P. S. Paul, K. A. Pomeroy, D. W. Johnson, C. C. Muscoplat, M. J. Van Der Maaten, J. M. Miller, and D. K. Sorensen.** 1978. Frequency of lymphocytes bearing Fc receptors and surface membrane immunoglobulins in normal, persistent lymphocytotic and leukemic cows. Am. J. Vet. Res. **39:**45–49.
35. **Lagarias, D. M., and K. Radke.** 1989. Transcriptional activation of bovine leukemia virus in blood cells from experimentally infected, asymptomatic sheep with latent infections. J. Virol. **63:**2099–2107.
36. **Levy, D., L. Deshayes, A. L. Parodi, J. R. Stephenson, S. G. Devare, and R. V. Gilden.** 1977. Bovine leukemia virus specific antibodies among French cattle. II. Radioimmunoassay with the major structural protein (BLV p24). Int. J. Cancer **20:**543–550.
37. **Lewin, H. A., M. C. Wu, J. A. Stewart, and T. J. Nolan.** 1988. Association between *BoLA* and subclinical bovine leukemia virus infection in a herd of Holstein-Friesian cows. Immunogenetics **27:**338–344.
38. **Mackay, C. R., J. F. Maddox, K. J. Gogolin-Ewens, and M. R. Brandon.** 1985. Characterization of two sheep lymphocyte differentiation antigens, SBU-T1 and SBU-T6. Immunology **55:**729–737.
39. **Mamoun, R. Z., M. Morisson, N. Rebeyrotte, B. Busetta, D. Couez, R. Kettmann, M. Hospital, and B. Guillemain.** 1990. Sequence variability of bovine leukemia virus *env* gene and its relevance to the structure and antigenicity of the glycoproteins. J. Virol. **64:**4180–4188.
40. **Matheise, J. P., M. Delcommenne, A. Mager, C. H. Didembourg, and J. J. Letesson.** 1992. CD5+ B cells from bovine leukemia virus infected cows are activated cycling cells responsive to interleukin 2. Leukemia **6:**304–309.
41. **Muscoplat, C. C., I. Alhaji, D. W. Johnson, K. A. Pomeroy, J. M. Olson, V. L. Larson, J. B. Stevens, and D. K. Sorenson.** 1974. Characteristics of lymphocyte responses to phytomitogens: comparison of responses of lymphocytes from normal and lymphocytotic cows. Am. J. Vet. Res. **35:**1053–1055.
42. **Parodi, A. L., H. Leveziel, E. Cribiu, D. Levy, and A. Vuillaume.** 1988. Genetic variability of resistance to enzootic bovine leukosis (a model), p. 565–576. 3rd World Conference on Sheep and Beef Cattle. Institut National de la Recherche Agronomique, Paris.
43. **Portetelle, D., M. Mammerickx, and A. Burny.** 1989. Use of two monoclonal antibodies in an ELISA test for the detection of antibodies to bovine leukaemia virus envelope protein gp51. J. Virol. Methods **23:**211–222.
44. **Powers, M. A., D. Grossman, L. C. Kidd, and K. Radke.** 1991. Episodic occurrence of antibodies against the bovine leukemia virus Rex protein during the course of infection in sheep. J. Virol. **65:**4959–4965.
45. **Puri, N. K., C. R. Mackay, and M. R. Brandon.** 1985. Sheep lymphocyte antigens (OLA). II. Major histocompatibility complex class II molecules. Immunology **56:**725–733.
46. **Rice, N. R., R. M. Stephens, A. Burny, and R. Gilden.** 1985. The *gag* and *pol* genes of bovine leukemia virus: nucleotide sequence and analysis. Virology **142:**357–377.
47. **Rice, N. R., R. M. Stephens, D. Couez, J. Deschamps, R. Kettmann, A. Burny, and R. Gilden.** 1984. The nucleotide sequence of the *env* and post-*env* region of bovine leukemia virus. Virology **138:**82–93.
48. **Rosen, C. A., J. Sodroski, R. Kettmann, A. Burny, and W. Haseltine.** 1984. Trans activation of the bovine leukemia virus long terminal repeat in BLV-infected cells. Science **227:**320–322.

49. **Rosenthal, S., B. Drescher, S. Weber, and R. Mohring.** 1990. Nucleotide sequence analysis of the 3′ half of the genome of bovine leukemia virus grown in FLK cells. Acta Virol. **34:**1–10.
50. **Rovnak, J., J. W. Casey, A. L. Boyd, M. A. Gonda, and G. L. Cockerell.** 1991. Isolation of bovine leukemia virus-infected endothelial cells from cattle with persistent lymphocytosis. Lab. Invest. **65:**192–202.
51. **Sagata, N., J. Tsuzuku-Kawamura, M. Nagayoshi-Aida, F. Shimizu, K. I. Imagawa, and Y. Ikawa.** 1985. Identification of some biochemical properties of the major X_{BL} gene product of bovine leukemia virus. Proc. Natl. Acad. Sci. USA **82:**7879–7883.
52. **Sagata, N., T. Yasunaga, J. Tsuzuku-Kawamura, K. Ohishi, Y. Ogawa, and Y. Ikawa.** 1985. Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses. Proc. Natl. Acad. Sci. USA **82:**677–681.
53. **Takashima, I., and C. Olson.** 1980. Effect of mitogens and anti-bovine leukosis serums on DNA synthesis of lymphocytes from cattle. Eur. J. Cancer **16:**639–645.
54. **Van Den Broeke, A., Y. Cleuter, G. Chen, D. Portetelle, M. Mammerickx, D. Zagury, M. Fouchard, L. Coulombel, and R. Kettmann.** 1988. Even transcriptionally competent proviruses are silent in bovine leukemia virus-induced sheep tumors. Proc. Natl. Acad. Sci. USA **85:**9263–9267.
55. **Van Der Maaten, M. J., and J. M. Miller.** 1976. Replication of bovine leukemia virus in monolayer cell cultures. Bibl. Haematol. **43:**360–362.
56. **Ward, W. H., C. K. Dimmock, and F. W. Eaves.** 1992. T lymphocyte responses of sheep to bovine leukaemia virus infection. Immunol. Cell Biol. **70:**329–336.
57. **Willems, L., R. Kettmann, F. Dequiedt, D. Portetelle, V. Voneche, I. Cornil, P. Kerkhofs, A. Burny, and M. Mammerickx.** 1993. In vivo infection of sheep by bovine leukemia virus mutants. J. Virol. **67:**4078–4085.
58. **Willems, L., D. Portetelle, P. Kerkhofs, G. Chen, A. Burny, M. Mammerickx, and R. Kettmann.** 1992. *In vivo* transfection of bovine leukemia provirus into sheep. Virology **189:**775–777.
59. **Willems, L., E. Thienpont, P. Kerkhofs, A. Burny, M. Mammerickx, and R. Kettmann.** 1993. Bovine leukemia virus, an animal model for the study of intrastrain variability. J. Virol. **67:**1086–1089.
60. **Yang, T. J., W. C. D. Hare, and D. A. Abt.** 1967. DNA and RNA synthesis in cultures of peripheral blood mononuclear leukocytes from normal and lymphosarcomatous cows. Br. J. Haematol. **13:**855–861.