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In Vivo Leukocyte Tropism of Bovine Leukemia Virus in Sheep and Cattle

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Bovine leukemia virus (BLV), an oncovirus related to human T-cell leukemia virus type I, causes a B-cell lymphoproliferative syndrome in cattle, leading to an inversion of the T-cell/B-cell ratio and, more rarely, to a B-cell lymphosarcoma. Sheep are highly sensitive to BLV experimental infection and develop B-cell pathologies similar to those in cattle in 90% of the cases. BLV tropism for B cells has been well documented, but the infection of other cell populations may also be involved in the BLV-induced lymphoproliferative syndrome. We thus looked for BLV provirus in other leukocyte populations in sheep and cattle by using PCR. We found that while B cells harbor the highest proviral load, $CD8^+$ T cells, monocytes, and granulocytes, but not $CD4^+$ T cells, also bear BLV provirus. As previously described, we found that persistent lymphocytosis in cows is characterized by an expansion of the $CD5^+$ B-cell subpopulation but we did not confirm this observation in sheep in which the expanded B-cell population expressed the CD11b marker. Nevertheless, BLV could be detected both in bovine $CD5^+$ and $CD5^-$ B cells and in sheep $CD11b^+$ and $CD11b^-$ B cells, indicating that the restricted BLV tropism for a specific B-cell subpopulation cannot explain its expansion encountered in BLV infection. Altogether, this work shows that BLV tropism in leukocytes is wider than previously thought. These results lead the way to further studies of cellular interactions among B cells and other leukocytes that may intervene in the development of the lymphoproliferative syndrome induced by BLV infection.

Bovine leukemia virus (BLV) infection induces a B-cell chronic lymphoproliferative syndrome in cattle. Seventy percent of infected animals present an expansion of the mature, immunoglobulin (Ig)-bearing B-lymphocyte population, and 1 to 5% develop a B-cell lymphosarcoma after a 1- to 8-year latency period (33). In Holstein cattle, the persistent B lymphocytosis is associated with a decline in milk and fat production (5). Infection of sheep with BLV represents an interesting experimental model to study BLV pathogenesis, because sheep are highly sensitive to experimental inoculation with BLV and develop B-cell lymphocytosis and B-cell lymphosarcomas in 90% of the cases after 1 to 4 years (35).

BLV is related to the human T-cell leukemia virus type I (HTLV-I), the causal agent of adult T-cell leukemia and lymphoma and of tropical spastic paraparesis. These retrovirus-induced pathologies are characterized by an absence of viremia, a rather long latency period, and a lack of preferred integration sites in tumors (4). BLV and HTLV-I share homologies in their genomic organization and present open reading frames between the *env* coding region and the 3′ long terminal repeat, two of them encoding the Tax and Rex proteins that are involved in the regulation of viral expression. Several pieces of experimental evidence suggest that the Tax and Rex proteins may play an important role in these virus-induced pathogeneses. The HTLV-I and BLV Tax proteins, although virus specific, transactivate their respective viral long terminal repeats through a cyclic AMP-responsive element (27). In addition, the Tax protein of HTLV-I has been shown to transactivate several cellular genes such as interleukin 2 (IL-2) (36), IL-2 receptor (3, 24), granulocyte macrophage colony-stimulating factor (40), c-*myc* (11), c-*fos* (16), tumor necrosis factor alpha (1), transforming growth factor beta (29), and vimentin (34). Infection of human cord blood cells with herpesvirus saimiri recombinants containing Tax coding sequences immortalizes $CD4^+$ T cells (17), and transfection of expression vectors for the Tax of BLV immortalizes rat embryo fibroblasts (44). The HTLV-I and BLV Rex products control the viral mRNA processing and modulate the accumulation of full-length and single-spliced mRNA (8, 22). The Rex protein of HTLV-I also stabilizes the IL-2 mRNA (25) and cooperates with the Tax protein of HTLV-I on the transactivation of IL-2 expression through the CD28-responsive element (36). However, the relevant molecular and cellular mechanisms by which HTLV-I induces $CD4^+$ T-cell leukemia and BLV alters B-cell homeostasis are still conjectural.

The tropism of these viruses for specific cell subpopulations is an important parameter to consider in order to understand their pathogenesis. HTLV-I has been shown to preferentially infect activated $CD4^+$ T cells ($CD45^-$ RO^+) and is also found integrated in vivo in $CD8^+$ T cells and granulocytes (41). In addition, HTLV-I can infect monocytes and microglial cells in primary cultures (23). How these infected cellular counterparts interact in the development of the disease is not known and deserves intensive investigation.

BLV is known to infect B lymphocytes in sheep and cattle. Integrated BLV provirus has been demonstrated in DNA obtained from enriched B cells derived from peripheral blood leukocytes (PBLs) of cattle and sheep with persistent lymphocytosis and lymphosarcomas (28, 32). In addition, BLV has been detected in mitogen-stimulated CD4 and CD8 cells derived from 7 of 10 lymphocytotic cattle (42). This finding could be the result of an in vitro infection of T cells and may not have any in vivo relevance. Furthermore, fluorescence-

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activated cell sorter (FACS)-purified T cells were found devoid of integrated BLV by Southern blot analysis, whereas cells characterized as monocytes were found infected (21).

In order to further clarify the issue of BLV cell tropism, we isolated highly purified leukocyte populations from infected sheep and cattle and used PCR to detect BLV provirus. We report herein that BLV is preferentially associated with B cells and that granulocytes, monocytes, and $CD8^+$ T cells, but not $CD4^+$ T cells, are also targets for BLV in vivo. We also found that, although BLV-induced lymphocytosis in cattle is characterized by an expansion of the B-cell population expressing the CD5 molecule (7, 31), BLV could be detected in both $CD5^+$ and $CD5^-$ B cells.

These data show that, as observed in HTLV-I (41), human immunodeficiency virus (13), and feline immunodeficiency virus (12) infections, BLV presents a preferential association with a specific cell type but that its in vivo tropism is wider than previously thought. The presence of the virus in these cell subsets may play a role in the development of the lymphoproliferative disease and the altered production traits encountered in cattle with BLV infection.

MATERIALS AND METHODS

BLV-infected sheep and cows and cell line. In 1988, six 6-month-old castrated male prealpine sheep were inoculated intraperitoneally with 50×10^6 lymphocytes from a BLV-infected cow with persistent lymphocytosis. Every animal became serologically positive within 4 weeks postinfection. Four years later, four of the six sheep had died with lymphosarcoma before the onset of our experiment. In the course of our studies, we used the remaining sheep, sheep 188, which had elevated lymphocyte counts (9,600 lymphocytes per mm^3 of whole blood, with 80% of the peripheral blood mononuclear cells [PBMCs] being B lymphocytes), and sheep 1011, which presented normal hematological parameters (3,200 lymphocytes per mm^3 of whole blood, with 25% of the PBMCs being B lymphocytes) and did not develop tumors. In March 1992, eight 6-month-old castrated male prealpine sheep were inoculated intraperitoneally with 50×10^6 lymphocytes from sheep 188. PCR analysis revealed that they were all BLV infected 4 weeks postinfection. One year later, seven of eight sheep were still hematologically normal, and sheep 130 showed a persistent inverted T-cell/B-cell ratio and slightly elevated lymphocyte counts (7,000/mm^3 of whole blood, with 76% of the PBMCs being B lymphocytes). These sheep were housed at the Ecole Nationale Vétérinaire, Maisons-Alfort, France.

Cows 206 and J047 had been found naturally infected with BLV. Cow 206 was in persistent lymphocytosis (16,200 lymphocytes per mm^3 of whole blood, with 78% of the PBMCs being B lymphocytes) and cow J047 was hematologically normal (2,300 lymphocytes per mm^3, with 24% of the PBMCs being B lymphocytes). Both cows were housed at the Centre National d'Etudes Vétérinaires et Alimentaires, Lyon, France.

The YR2 cell line was established from a BLV-induced sheep lymphosarcoma and was kindly provided by L. Droogmans (Université Libre de Bruxelles, Brussels, Belgium).

MAbs and flow cytometry analysis. Unless a specific reference is mentioned, the monoclonal antibodies (MAbs) used in this study were reported at the Workshop on Leukocyte Antigens in Cattle, Sheep and Goats, Hannover, Germany, 1989 (23a).

Four anti-sheep lymphocyte MAbs were kindly provided by W. Hein, Basel Institute for Immunology, Basel, Switzerland. The IgG2a ST-1A, IgM ST-8, and IgG1 ST-4 MAbs define the sheep CD5, CD8, and CD4 homologs, respectively. The IgM DU2-104 MAb specifically labels all of the surface Ig-bearing cells and as such is considered to be an anti-B-cell MAb. Although the molecular determinant that it recognizes is unknown, DU2-104 is not an anti-Ig MAb (21a).

Most of the MAbs used to label and sort bovine lymphocytes were obtained from the International Laboratory for Research on Animal Disease, Nairobi, Kenya. We used the IgG2a IL-A11, IgG1 IL-A15, IgM IL-A28, and IgG1 IL-A24 MAbs that, respectively, define CD4, CD11b, CD6, and a myeloid marker expressed on monocytes and granulocytes. The IL-A105 and the VC13 MAbs define the bovine CD8 and CD1 homologs, respectively (34a). The CC17 hybridoma (IgG1) that defines the bovine CD5 homolog was obtained from the Institute of Animal Health, Compton, Great Britain.

For flow cytometry analysis, 2×10^6 PBLs were incubated for 30 min in 50 μl of hybridoma culture supernatant at 4°C, washed twice in phosphate-buffered saline (PBS) with 2% filtered horse serum, and incubated for 30 min in 50 μl of a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-mouse IgG1 (Caltag Laboratories, San Francisco, Calif.) or phycoerythrin (PE)-conjugated $F(ab')_2$ goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, Pa.). After three washes, the cells were analyzed with a Facs Scan (Becton Dickinson, Mountain View, Calif.). The FITC-conjugated rabbit anti-bovine IgG heavy and light chains were purchased from Jackson ImmunoResearch Laboratories.

Separation of leukocyte subsets with immunomagnetic beads. PBLs (50×10^6) were incubated with M450 beads (Dynal, Great Neck, N.Y.) coated with MAbs to specific cell subpopulations at a bead/cell ratio of 5:1 for 1 h under agitation at 4°C. As a control in sheep cell-sorting experiments, M450 beads were coated with the IL-A28 MAb that does not recognize any sheep determinant. As a control in bovine cell sorting, beads were coated with the VC13 MAb that recognizes the CD1 molecule, which is normally not expressed on PBLs. The cell-coated beads were washed 20 times with 4 ml of PBS plus 0.1% bovine serum albumin (BSA), checked under light microscopy for the absence of free cells on a whole Malassez grid, and lysed with 10 μl of lysis buffer per 2×10^4 cells (10 mM Tris-HCl [pH 8], 50 mM KCl, 20 μg of BSA per ml, 0.45% Tween 20, 0.45% Nonidet P-40). The lysate was then subjected to a 200-μg/ml proteinase K treatment at 50°C for 1 h, heated at 94°C for 5 min, and analyzed by PCR.

For controlling the purity of the recovered cell subsets, 10^5 immunomagnetic bead-purified cells were incubated in 100 μl of PBS and 1 U of DETACHaBEAD (Dynal) for 2 h. The detached cells were then incubated for 30 min with 100 μg of mouse Igs to adsorb the DETACHaBEAD reagent, washed three times in PBS plus 2% filtered horse serum, incubated overnight at 37°C, and immunolabelled for flow cytometry as described above.

FACS of leukocyte subpopulations. For sorting sheep monocytes and granulocytes, 10^8 Percoll-enriched PBMCs or polymorphonuclear cells were first partially depleted with DU2-104- and ST-1A-coated M450 beads at a bead/cell ratio of 10:1. The partially B- and T-depleted cells (25×10^6) were incubated for 30 min in 500 μl of IL-A24 and 500 μl of DU2-104 culture supernatants, washed twice, and incubated for 30 min in 250 μl of a 1:50 dilution of FITC-conjugated $F(ab')_2$ goat anti-mouse IgG1 and PE-conjugated $F(ab')_2$ goat anti-mouse IgM. The cells were then washed three times, and 10^5 $IL\text{-}A24^+$ $DU2\text{-}104^-$ cells were sorted according to their side-scatter index at a rate of 2,000 cells per s with a Facs Sort (Becton Dickinson). A sample of the sorted cells was checked by FACS and found to be >98% pure.

For sorting ovine CD11b$^+$ and CD11b$^-$ B cells, 25×10^6 Percoll-purified PBMCs were labelled with 250 μl of DU2-104 culture supernatant and a 1:100 dilution of IL-A15 mouse ascites. For sorting bovine CD5$^+$ and CD5$^-$ B cells, 25×10^6 PBMCs were incubated with 250 μl of DU2-104 and 250 μl of CC17 culture supernatant. The cells were washed twice, incubated with 250 μl of a 1:50 dilution of FITC-conjugated F(ab')$_2$ goat anti-mouse IgG1 and PE-conjugated F(ab')$_2$ goat anti-mouse IgM, and washed three times, and 10^5 cells were sorted at a rate of 2,000 cells per s with a Facs Sort. The sorting efficiency was checked by FACS, and the recovered cells were >98% pure.

Detection of BLV provirus by PCR and Southern blotting. PCR amplification and Southern blot hybridization with primers and a probe specific for the Tax coding region were used to detect BLV provirus. The cell lysates (10 μl) corresponding to 2×10^4 cells from each sorted leukocyte population were subjected to PCR analysis. Two sets of primers were used in each PCR. One set amplifies a 283-bp segment encompassing part of the Tax coding region with an upstream primer, BLV-B1 (5'-GAT GCC TGG TGC CCC CTC TG), and a downstream primer, BLV-B2 (5'-ACC GTC GCT AGA GGC CGA GG); the other set amplifies a 160-bp fragment from the sheep growth hormone gene and constitutes the NOOGH 1 upstream primer (5'-CGG GTG GCA GTG GAG AGG GGA TGA T) and the NOOGH 2 downstream primer (5'-TCT CCC TTT CCC CCC ACC TGT GTG C). NOOGH 1 and NOOGH 2 amplify the same length of fragment in the cow. The amplified sheep growth hormone gene fragment attests to efficient PCR and is revealed by ethidium bromide staining. PCR amplification was performed in 10 mM Tris-HCl (pH 8)–50 mM KCl–20 μg of BSA per ml–1.5 mM $MgCl_2$–100 pmol of each primer–200 μM deoxynucleoside triphosphate–1.6 U of *Taq* polymerase (Appligène, Strasbourg, France) in a final 100-μl reaction mixture. An amplification at 91°C for 1 min and 71°C for 4 min was repeated 30 times and was followed by a 10-min 71°C extension cycle. Fifteen microliters of amplified product was run on a 1.8% agarose gel that was transferred after denaturation to a nylon membrane (Amersham-France, Les Ulis, France). Membranes were hybridized at 42°C either with a ^{32}P- or digoxigenin-labelled *tax* probe generated from an *Xba*I-*Pvu*II insert encompassing the entire Tax sequence (43). After one wash in 1× SSC (0.15 M NaCl and 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS) at 65°C for 15 min and one wash in 0.1× SSC plus 0.1% SDS at 65°C for 10 min, blots were either exposed to a radiographic film or, in the case of the digoxigenin-labelled probe, were further processed according to the manufacturer's recommendations (Boehringer Mannheim GmbH, Mannheim, Germany). The Tax-specific primers and probe did not recognize any sequences from normal sheep or cow PBLs.

RESULTS

Detection of BLV provirus in lymphocyte subsets of BLV experimentally infected sheep. In order to assess the in vivo tropism of BLV in experimentally infected sheep, it was necessary to obtain highly purified subpopulations of lymphocytes. CD4$^+$, CD8$^+$, and B-lymphocyte subpopulations were purified with magnetic beads coated with the ST-4, ST-8, and DU2-104 MAbs, respectively, and the recovered cell subpopulations were >95% pure by FACS analysis (data not shown). A minimum of 10^5 purified cells were sorted with the MAb-coated beads. A total of 2×10^4 lysed cells were analyzed with PCR primers amplifying a 283-bp internal segment of the Tax coding region, followed by Southern blotting and hybridization with a Tax-specific probe. To verify the efficiency of the amplification process in each reaction, we performed PCR amplification of a fragment derived from the sheep growth hormone gene. In addition, a control with magnetic beads coated with an antibody to bovine CD6 that does not cross-react with any sheep determinant was included in each sorting procedure.

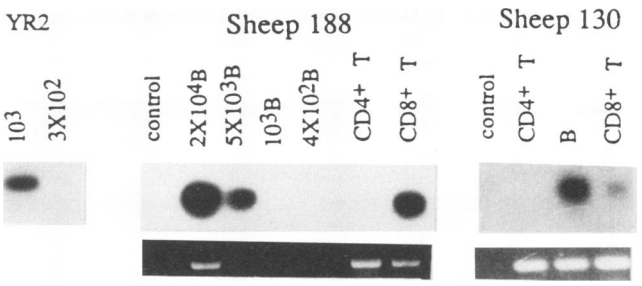

FIG. 1. Detection of BLV provirus in sheep 188 lymphocytes, in sheep 130 lymphocytes, and in the YR2 cell line. PCR amplification of lysates from purified lymphocyte subpopulations was performed with the Tax primers BLV B1 and BLV B2, and subsequent Southern Blotting and probing were performed with a Tax-specific probe. Totals of 10^3 and 3×10^2 cell lysate equivalents from the YR2 cell line that carries a single copy of integrated BLV provirus were included for quantitation. Control lanes contain lysates obtained from a control sorted with the anti-bovine MAb CD6, which does not recognize any sheep determinant. B cells and CD4$^+$ and CD8$^+$ cells were purified with DU2-104-, ST-4-, and ST-8-coated immunomagnetic beads, respectively. Unless otherwise mentioned, lysates were made of 2×10^4 cells. At the bottom, ethidium bromide staining of a PCR-amplified fragment derived from the sheep growth hormone gene is shown. The sheep growth hormone gene limit of detection is reached with 5×10^3 cells.

We first analyzed the purified subpopulation of sheep 188 that had been infected with BLV for 4 years and showed a B-cell-persistent lymphocytosis. As illustrated in Fig. 1, BLV provirus could easily be detected in CD8$^+$ T and B lymphocytes. Interestingly, the signal for BLV integration in the CD4$^+$ T cells was undetectable with our technique. A serial dilution of the DU2-104-sorted population showed that the signal intensity obtained with 5×10^3 B cells was equivalent to the signal obtained with 10^3 cells from the YR2 cell line, a cell line bearing a single integrated copy of the BLV genome per cell. Assuming that the BLV-infected lymphocytes bear one BLV provirus per cell, these data show that the ratio of BLV-bearing B cells to BLV-nonbearing B cells is around 1:5 in sheep 188 B lymphocytes, a value in agreement with a previously published estimate (28). The faint signal obtained with lysate from 3×10^2 YR2 cells indicates the limit of sensitivity of our technique (around 1 to 2 infected cells per 100); this rather limited PCR efficiency was mandatory not to detect the infected contaminating cells that are inherent because of the purity of the sorting procedure. Consequently, the undetectability of BLV provirus in CD4$^+$ T cells means that <1 of 100 CD4$^+$ T cells is infected with BLV, whereas comparative signal intensities between 10^3 YR2 and 2×10^4 CD8$^+$ T cells indicate that >5 of 100 CD8$^+$ T cells are infected in sheep 188. Finally, because no signal could be obtained with 10^3 B cells, this indicates that a 5% B-cell contamination in the CD8$^+$ T-cell sample cannot account for the signal obtained with CD8$^+$ T cells and attests to the reliability of BLV detection in the CD8$^+$ T cells.

To ascertain that BLV provirus in CD8$^+$ T cells of sheep 188 was not a feature particular to that sheep, we performed a

TABLE 1. Detection of BLV provirus in sheep leukocyte subpopulations

Sheep	T-cell/B-cell ratio[a]	BLV provirus detection[b]			
		B cells	$CD8^+$ T cells	$CD4^+$ T cells	IL-A24
188	0.17	+	+	−	+
130	0.21	+	+	−	+
121	1.25	+	+	−	+
142	3.3	+	+	−	+
131	1.4	+	+	−	−
105	1.4	+	+	−	−
120	5.0	+	−	−	+
122	1.2	+	−	−	−
135	2.5	+	−	−	−

[a] A T-cell/B-cell ratio was estimated by using double-fluorescence labelling with the sheep anti-CD5 (ST-1A) and the anti-B (DU2-104) MAbs. A T-cell/B-cell ratio of >1.2 is considered to be normal.
[b] +, detected; −, not detected.

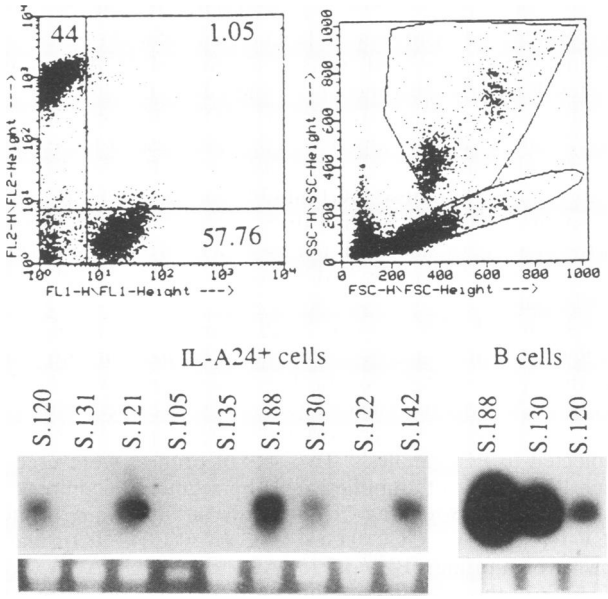

FIG. 2. Detection of BLV provirus in myeloid leukocytes. (Top left) Two-color flow cytometry analysis of sheep 130 Percoll-purified PBLs labelled with the DU2-104 MAb (FL2-H axis [PE]) and IL-A24 MAb (FL1-H axis [FITC]). The quadrants were traced on the basis of a control staining with the PE- and FITC-conjugated anti-mouse antibodies alone. Numbers represent the percentage of positively stained PBLs in each quadrant. (Top right) Forward-to-side-scatter profile of sheep 130 PBLs. The polygon corresponds to the IL-A24-labelled cells; the ellipse corresponds to the DU2-104-labelled cells. (Bottom left) Each lane corresponds to the Tax PCR product obtained with $IL\text{-}A24^+$ immunomagnetic bead-purified cells from nine sheep (symbolized by S. followed by the sheep number). (Bottom right) Corresponding Tax PCR signals obtained with the B-cell lysates from three sheep. At the bottom, ethidium bromide staining of a PCR-amplified fragment derived from the sheep growth hormone gene is shown.

similar experiment with PBMCs of sheep 130, which had been infected for 1 year and showed a mild lymphocytosis with an inverted B-cell/T-cell ratio. We obtained a profile of BLV tropism similar to the one obtained with sheep 188, with no detectable BLV in the $CD4^+$ T-cell population and detection of BLV provirus in the B-cell and $CD8^+$ T-cell populations (Fig. 1).

We extended our experiments to seven other sheep that had been infected for 1 year and that, to date, showed no obvious hematological perturbations. Four sheep showed the same BLV tropism profile as sheep 130 and sheep 188. However, the three other sheep presented detectable BLV only in B lymphocytes (Table 1).

Detection of BLV provirus in myeloid leukocytes from experimentally infected sheep. Monocytes and macrophages represent an important reservoir in human immunodeficiency virus (19), caprine arthritis-encephalitis virus (39), simian immunodeficiency virus (10), and equine infectious anemia virus (37) infections and are suspected to play a role in the development of the diseases they induce. We thus sought BLV in the monocytes of the BLV-infected sheep.

B lymphocytes from BLV-infected cows and sheep are known to express surface molecules that are usually expressed on myeloid cells (31). We screened a panel of MAbs known to characterize sheep or bovine myeloid cells in order to find an antibody that does not cross-react with B cells in BLV-infected sheep. Using double-fluorescence flow cytometry analysis with the IgM DU2-104 MAb, we were able to find that IgG1 IL-A24, a MAb to bovine myeloid cells, does not recognize B cells in our BLV-infected sheep and gives a side-to-forward-scatter profile that characterizes granulocytes and monocytes (Fig. 2).

We then performed magnetic bead sorting of Percoll-purified mononuclear cells by using the IL-A24 antibody and found that sheep 188, sheep 130, and three of seven of our hematologically normal sheep harbored detectable BLV provirus in the $IL\text{-}A24^+$ population (Fig. 2 and Table 1). In two hematologically normal sheep, BLV detection in the myeloid cells was associated with BLV detection in the $CD8^+$ T cells (Table 1).

Because the Percoll purification does not eliminate granulocytes, we then asked whether BLV was present in the monocytic or the granulocytic population. We sorted out these two populations by using a FACS. We first partially depleted the Percoll-purified mononuclear cells of B and T cells by using magnetic beads coated with the DU2-104 and anti-CD5 ST-1A MAbs in order to facilitate the sorting procedure. We labelled the partially B- and T-depleted cells with the IL-A24 and DU2-104 MAbs. As expected, no double-positive cells could be detected (Fig. 3). We then sorted the $IL\text{-}A24^+$ cells with a low-side-scatter index that are considered monocytes (Fig. 3A). We performed a similar sorting procedure with Percoll-enriched polymorphonuclear cells. We gated the $IL\text{-}A24^+$ cells with a high-side-scatter index that are considered granulocytes (Fig. 3B). The sorted cells were checked for purity by FACS analysis and were found to be >98% pure. This experiment, performed with sheep 188 and sheep 130 leukocytes, shows that both granulocytes and monocytes bear BLV provirus (Fig. 3C).

The spectrum of the BLV leukocyte target found in sheep is similar to that in a cow with persistent lymphocytosis. We set as our goal purification of leukocyte subpopulations from a cow with persistent lymphocytosis in order to see whether the spectrum of BLV tropism found in sheep was any different in cattle. We purified $CD4^+$, $CD8^+$, and B cells with magnetic beads coated with the IL-A11, IL-A105, and DU2-104 MAbs, respectively. Double-color flow cytometry analysis with FITC-conjugated rabbit anti-bovine IgG heavy and light chains and the PE-conjugated $F(ab')_2$ goat anti-mouse IgM showed that DU2-104 also recognizes the Ig-bearing lymphocytes in cattle (data not shown). Here again, we found that BLV was present in $CD8^+$ T and B cells and not in $CD4^+$ T cells (Fig. 4). We

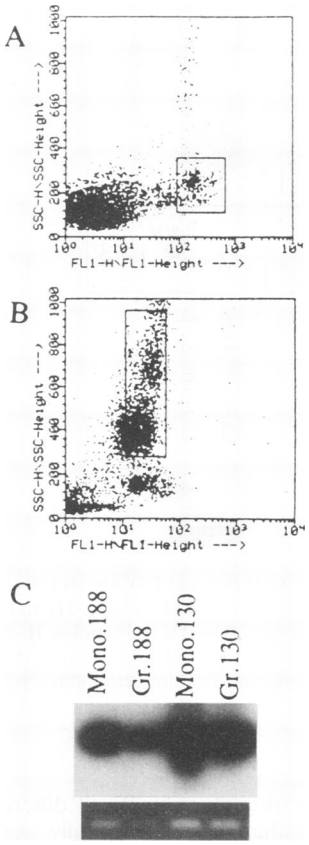

FIG. 3. Detection of BLV provirus in monocytes and granulocytes. (A) FL1-H-to-side-scatter profile of sheep 130 Percoll-enriched mononuclear cells labelled with IL-A24. The square corresponds to the monocytes (7.84% of the analyzed cells) that were sorted with a Facs Sort. (B) FL1-H-to-side-scatter profile of sheep 130 Percoll-enriched polymorphonuclear cells labelled with IL-A24. The rectangle corresponds to the granulocytes (24.8% of the analyzed cells) that were sorted with a Facs Sort. (C) Tax PCR products obtained with the sorted monocytes (Mono.) and granulocytes (Gr.) from sheep 188 and sheep 130. At the bottom, ethidium bromide staining of a PCR-amplified fragment derived from the sheep growth hormone gene is shown.

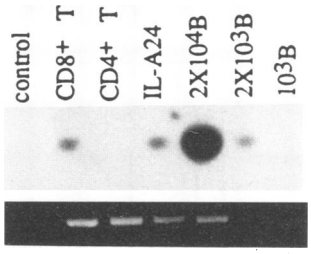

FIG. 4. Detection of BLV provirus in purified leukocyte subpopulations from cow 205. Unless otherwise mentioned, lanes represent the Tax PCR product corresponding to 2×10^4 cells. Control was obtained from a control sorted with the VC13 MAb, which recognizes dendritic cells that are usually absent in PBLs. B cells, $CD4^+$ and $CD8^+$ cells, and myeloid cells were isolated with DU2-104-, IL-A11-, IL-A105-, and IL-A24-coated immunomagnetic beads, respectively. At the bottom, ethidium bromide staining of a PCR-amplified fragment derived from the bovine growth hormone gene is shown.

also sorted the myeloid cells with the IL-A24 MAb after having checked that IL-A24 and DU2-104 define two independent populations with double-color flow cytometry analysis. As was found for sheep, integrated BLV could also be detected in the bovine myeloid leukocytes (Fig. 4).

BLV shows a preferential tropism for B cells that is not restricted to the B-cell subsets expanded in persistent lymphocytosis. Of all of the leukocyte subpopulations tested, B cells were the only population invariably showing the presence of BLV provirus. In addition, the signal attesting to BLV in B cells shown in Fig. 1, 2, and 3 was stronger than those in other leukocyte subpopulations. This indicates that BLV presents preferential tropism for B cells. We then wondered whether BLV would be associated with specific B-cell subsets. Previous reports mentioned that some of the B cells from BLV-infected sheep and cows express simultaneously high levels of IgM and CD5, a marker usually found on T lymphocytes, and that this $CD5^+$ B-cell population is expanded in persistent lymphocytosis (7, 30, 31). One suggested hypothesis (7, 31) was that BLV might show a restricted tropism for $CD5^+$ B cells that have been defined as $Ly1^+$ B cells in mice and may represent a separate B-cell subset (26). In order to test this hypothesis, we isolated $CD5^+$ and $CD5^-$ B lymphocytes from the lymphocytotic cow (cow 205). B cells were labelled with the IgM DU2-104 MAb, the $CD5^+$ cells were labelled with the IgG1 CC17 MAb, and CD5 was detected on 90% of the B cells (Fig. 5). We then sorted the $CD5^+$ and $CD5^-$ B cells, and we determined by FACS analysis that the purity of the recovered populations was >98%. We found that BLV was more prominent in the $CD5^+$ B-cell population than in the $CD5^-$ B-cell population (Fig. 5). Because this finding needed to be validated by extending it to other cows, we performed the same experiment with cow J047, which was hematologically normal. We found that 63% of the B cells expressed CD5, and in this case, BLV provirus was equally represented in the FACS-sorted $CD5^+$ and $CD5^-$ B cells (Fig. 5). Such an experiment could not be conducted with sheep because our BLV-infected sheep did not express CD5 on B cells. Because B cells in lymphocytotic cattle have also been shown to express myeloid markers (31), we tested whether B cells from the BLV-infected sheep presented any myeloid marker cell surface expression. We found that 12 to 90% of B cells in the BLV-infected sheep expressed the molecule CD11b that was detected with the IL-A15 MAb and that the proportion of $CD11b^+$ B cells was much higher in the two lymphocytotic sheep (90 and 89% in sheep 130 and 188, respectively, and 12 to 56% in the seemingly hematologically normal BLV-infected sheep). We sorted the $CD11b^+$ and the $CD11b^-$ B cells from sheep 130, determined that the purity of the recovered populations was >98% by FACS analysis, and found that both populations bore BLV provirus (Fig. 5). Altogether, these results invalidate the hypothesis of a restricted BLV tropism for the $CD5^+$ or $CD11b^+$ B cells among other B cells as a basis for the mechanism leading to the expansion of these subpopulations.

DISCUSSION

In BLV experimentally infected sheep and in one naturally infected cow with persistent lymphocytosis, we found that, in addition to the B-cell major target, $CD8^+$ T cells, granulocytes, and monocytes were infected. This broad leukocyte specificity is well reflected in vitro, because BLV has been found to infect a wide variety of cells, such as the canine osteosarcoma D17, human cervical carcinoma HeLa, and bovine kidney MDBK cell lines (9). This finding suggests that the BLV receptor is not restricted to a specific cell population but is rather broadly

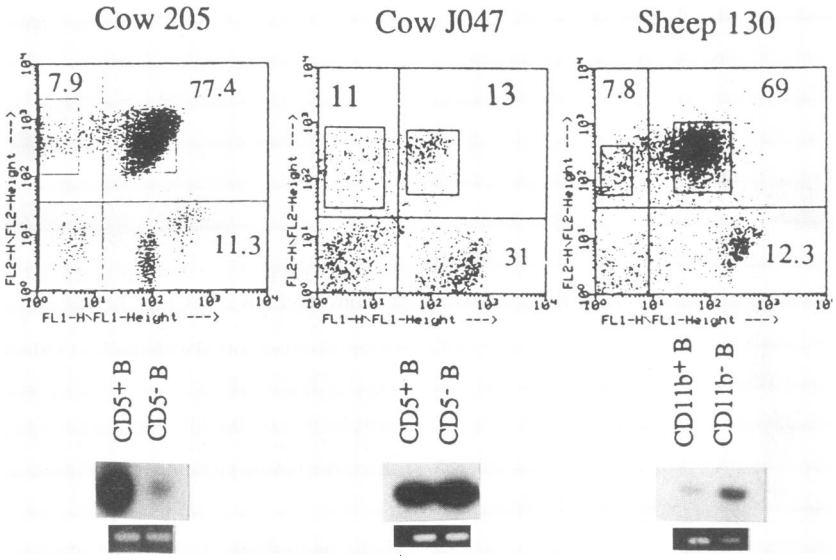

FIG. 5. Detection of BLV provirus in $CD5^+$ and $CD5^-$ B cells of cows 205 and J047 and in $CD11b^+$ and $CD11b^-$ B cells of sheep 130. (Top) FACS profiles (FL1-H to FL2-H) of cow 205 and cow J047 PBMCs labelled with the anti-B-cell DU2-104 MAb (FL2-H) and the anti-$CD5^+$ cell CC17 MAb (FL1-H) and of sheep 130 PBMCs labelled with the DU2-104 MAb (FL2-H) and the anti-CD11b homolog IL-A15 MAb (FL1-H). Numbers represent the percentage of positive cells in each quadrant. The rectangles indicate the populations that were sorted with a Facs Sort. Middle panels show Tax PCR products from cell lysates of 2×10^4 FACS-sorted cells. At the bottom, ethidium bromide staining of a PCR-amplified fragment derived from the growth hormone gene is shown.

expressed. However, BLV could not be detected in $CD4^+$ T cells. This nondetection, which could be the result of limits in the sensitivity of our technique, nevertheless indicates a relative viral inefficiency in penetration or complete viral reverse transcription or integration in $CD4^+$ T cells.

BLV infection of cell types other than B cells may have an impact on the development of the B-cell lymphoproliferative syndrome and the B-cell lymphosarcoma. Interestingly, sheep 1011 has been infected for 5 years, has kept a normal T-cell/B-cell ratio, has not developed tumors, and does not harbor BLV provirus in CD8 and myeloid cells (data not shown). This observation needs to be extended to other long-term-infected sheep in order to correlate the development of the B-cell lymphoproliferative syndrome with BLV infection in myeloid cells and $CD8^+$ T cells. Unfortunately, most of our sheep herd has been too recently infected to help establish such a correlation.

Whether or not BLV is expressed in $CD8^+$ T cells and myeloid cells is an important avenue to explore in order to assess the role of these cell targets in BLV-induced pathology. The alternatively spliced BLV mRNA can be detected in vivo in sheep total PBLs by reverse transcription and PCR (2, 18), and their specific expression in the purified leukocyte subpopulations could be analyzed. The expression of viral proteins in these cell subsets may trigger abnormal gene expression, such as production of ILs, and alter the cellular interactions normally occurring in homeostasis, leading to the expansion of the B-cell population.

Persistent lymphocytosis in BLV-infected sheep and cattle is characterized by an expansion of the B-cell population coexpressing CD5 and myeloid markers (7, 30, 31). $CD5^+$ B cells in cattle and humans are considered to be the equivalent of $Ly1^+$ murine B cells and express the usual B-cell markers as well as myeloid markers and an IgM^{high} IgD^{low} phenotype (14, 38). There is as yet no evidence to assess whether they constitute a different lineage of B cells with different precursors or whether they are an activation stage in B-cell differentiation (14, 15, 20). We showed that BLV was mainly associated with the $CD5^+$ B cells in a cow with persistent lymphocytosis, whereas BLV could be detected as well in both the $CD5^+$ and $CD5^-$ B cells of a seemingly hematologically normal BLV-infected cow. This discrepancy may reflect individual differences or may be explained by the higher proliferation rate of the $CD5^+$ B cells in lymphocytosis, because retroviruses preferentially integrate in dividing cells (45). Besides, a large proportion of the B-cell population in our BLV-infected sheep did not express the CD5 molecule but expressed the myeloid marker CD11b. There again, BLV was equally associated with $CD11b^+$ and $CD11b^-$ B cells.

This implies that a restricted tropism for the $CD5^+$ or $CD11b^+$ B cells cannot account for the expansion of these B-cell subpopulations encountered in BLV infection. In addition, the detection of CD11b and not CD5 on the B cells from our BLV-infected sheep is not in accordance with a previous report showing that B cells from BLV-infected sheep express CD5 (30). It can then be inferred that CD5 on B cells is not a specific marker associated with BLV infection and BLV-induced B-cell lymphocytosis in sheep. Furthermore, although $CD5^+$ B cells have been shown to also bear CD11b in mice and humans (6, 26), our data suggest that CD5 and CD11b expression can segregate in sheep.

The significance of the expression of CD5 or CD11b on the expanded B-cell population in BLV-infected animals is puzzling. $CD5^+$ or $CD11b^+$ B cells may show a particular susceptibility to proliferate and expand while infected with BLV. Alternatively, BLV expression may trigger the expression of CD5 and/or CD11b that may play a role in the development of the BLV-induced lymphocytosis. The Tax protein of HTLV-I has been shown to transactivate many cellular genes (1, 3, 11, 24, 29, 34, 40), and, similarly, the Tax protein of BLV may be involved in transactivating CD5 and/or CD11b expression.

The data presented herein show that BLV is preferentially

associated with B cells but exhibits no obvious preferred tropism for the $CD5^+$ and/or $CD11b^+$ B-cell subpopulations. In addition, BLV was found in $CD8^+$ T cells, monocytes, and granulocytes. One cannot exclude that other leukocytes, such as NK cells and γ/δ T cells, also carry BLV provirus. These infected subpopulations that seem unaffected by BLV infection may only be bystanders or may play an active role in the onset of the viral disease. Further analyses, such as RNA expression in different cell subsets, correlation of cell subset infection and symptoms, and in vitro studies of cell-cell interactions, may help us define the role of BLV infection in specific cell types to better understand how cellular interactions participate in the development of BLV-induced pathology.

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REFERENCES

1. **Albrecht, H., A. N. Shakhov, and C. V. Jongeneel.** 1992. *trans* activation of the tumor necrosis factor alpha promoter by the human T-cell leukemia virus type I Tax_1 protein. J. Virol. **66:**6191–6193.
2. **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.
3. **Ballard, D. W., E. Bohnlein, J. W. Lowenthal, Y. Wano, B. R. Franza, and W. C. Greene.** 1988. HTLV-I Tax induces cellular proteins that activate the κ B element in the IL-2 receptor α gene. Science **241:**1652–1655.
4. **Burny, A., Y. Cleuter, R. Kettmann, M. Mammerickx, G. Marbaix, D. Portetelle, A. Van den Broeke, L. Willems, and R. Thomas.** 1988. Bovine leukemia: facts and hypotheses derived from the study of an infectious cancer. Adv. Vet. Sci. Comp. Med. **32:**149–170.
5. **Da, Y., R. D. Shanks, J. A. Stewart, and H. A. Lewin.** 1993. Milk and fat yields decline in bovine leukemia virus-infected Holstein cattle with persistent lymphocytosis. Proc. Natl. Acad. Sci. USA **90:**6538–6541.
6. **De la Hera, A., M. Alvarez Mon, F. Sanchez Madrid, C. Martinez, and A. Durantez.** 1988. Co-expression of Mac-1 and p150,95 on CD5+ B cells. Structural and functional characterization in a human chronic lymphocytic leukemia. Eur. J. Immunol. **18:**1131–1134.
7. **Depelchin, A., J. J. Letesson, N. Lostrie Trussart, M. Mammerickx, D. Portetelle, and A. Burny.** 1989. Bovine leukemia virus (BLV)-infected B-cells express a marker similar to the CD5 T cell marker. Immunol. Lett. **20:**69–76.
8. **Derse, D.** 1988. *trans*-acting regulation of bovine leukemia virus mRNA processing. J. Virol. **62:**1115–1119.
9. **Derse, D., and L. Martarano.** 1990. Construction of a recombinant bovine leukemia virus vector for analysis of virus infectivity. J. Virol. **64:**401–405.
10. **Desrosiers, R. C., A. Hansen-Moosa, K. Mori, D. P. Bouvier, N. W. King, M. D. Daniel, and D. J. Ringler.** 1991. Macrophage tropic v. riants for SIV are associated with specific AIDS-related lesions but are not essential for the development of AIDS. Am. J. Clin. Pathol. **139:**29–35.
11. **Duyao, M. P., D. J. Kessler, D. B. Spicer, and G. E. Sonenshein.** 1992. Transactivation of the murine c-myc gene by HTLV-1 tax is mediated by NFκB. AIDS Res. Hum. Retroviruses **8:**752–754.
12. **English, R. V., C. M. Johnson, D. H. Gebhard, and M. B. Tompkins.** 1993. In vivo lymphocyte tropism of feline immunodeficiency virus. J. Virol. **67:**5175–5186.
13. **Fauci, A. S.** 1988. The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. Science **239:**617–622.
14. **Freedman, A. S., G. Freeman, J. Whitman, J. Segil, J. Daley, H. Levine, and L. M. Nadler.** 1989. Expression and regulation of CD5 on *in vitro* activated human B cells. Eur. J. Immunol. **19:**849–855.
15. **Freedman, A. S., G. Freeman, J. Whitman, J. Segil, J. Daley, and L. M. Nadler.** 1989. Studies of *in vitro* activated CD5+ B cells. Blood **73:**202–208.
16. **Fuji, M., P. Sassone-Corsi, and I. M. Verma.** 1993. C-fos promoter transactivation by the Tax protein of human T cell leukemia virus type 1. Proc. Natl. Acad. Sci. USA **85:**8526–8530.
17. **Grassmann, R., S. Berchtold, I. Radant, M. Alt, B. Fleckenstein, J. G. Sodroski, W. A. Haseltine, and U. Ramstedt.** 1992. Role of human T-cell leukemia virus type 1 X region proteins in immortalization of primary human lymphocytes in culture. J. Virol. **66:**4570–4575.
18. **Haas, L., T. Divers, and J. W. Casey.** 1992. Bovine leukemia virus gene expression in vivo. J. Virol. **66:**6223–6225.
19. **Hattori, N., F. Michaels, K. Fargnoli, L. Marcon, R. C. Gallo, and G. Franchini.** 1990. The human immunodeficiency virus type 2 vpr is essential for productive infection of human macrophages. Proc. Natl. Acad. Sci. USA **87:**8080–8084.
20. **Hayakawa, K., R. R. Hardy, and L. A. Herzenberg.** 1985. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. J. Exp. Med. **161:**1554–1568.
21. **Heeney, J. L., P. J. Valli, R. M. Jacobs, and V. E. 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.

21a. **Hein, W.** Personal communication.

22. **Hidaka, M., J. Inoue, M. Yoshida, and M. Seiki.** 1988. Posttranscriptional regulator (rex) of HTLV-1 initiates expression of viral structural proteins but suppresses expression of regulatory proteins. EMBO J. **7:**519–523.
23. **Hoffman, P. M., S. Dhib Jalbut, J. A. Mikovits, D. S. Robbins, A. L. Wolf, G. K. Bergey, N. C. Lohrey, O. S. Weislow, and F. W. Ruscetti.** 1992. Human T cell leukemia virus type I infection of monocytes and microglial cells in primary human cultures. Proc. Natl. Acad. Sci. USA **89:**11784–11788.

23a. **Howard, C. J., and W. I. Morrison (ed.).** 1991. Leukocyte antigens in cattle, sheep and goats. Vet. Immunol. Immunopathol. **27.**

24. **Inoue, J., M. Seiki, T. Taniguchi, S. Tsuru, and M. Yoshida.** 1986. Induction of interleukin 2 receptor gene expression by p40x encoded by human T cell leukemia virus type 1. EMBO J. **5:**2883–2888.
25. **Kanamori, H., N. Suzuki, H. Siomi, T. Nosaka, A. Sato, H. Sabe, M. Hatanaka, and T. Honjo.** 1990. HTLV-1 p27rex stabilizes human interleukin-2 receptor alpha chain mRNA. EMBO J. **9:**4161–4166.
26. **Kantor, A. B.** 1991. The development and repertoire of B-1 cells (CD5 B cells). Immunol. Today **12:**389–391.
27. **Katoh, I., Y. Yoshinaka, and Y. Ikawa.** 1989. Bovine leukemia virus trans-activator p38tax activates heterologous promoters with a common sequence known as a cAMP-responsive element or the binding site of a cellular transcription factor ATF. EMBO J. **8:**497–503.
28. **Kettmann, R., Y. Cleuter, M. Mammerickx, M. Meunier-Rotival, G. Bernardi, A. Burny, and H. Chantrenne.** 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.
29. **Kim, S. J., J. H. Kehrl, J. Burton, C. L. Tendler, K. T. Jeang, D. Danielpour, C. Thevenin, K. Y. Kim, M. B. Sporn, and A. B. Roberts.** 1990. Transactivation of the transforming growth factor beta 1 (TGF-beta 1) gene by human T lymphotropic virus type 1 Tax: a potential mechanism for the increased production of TGF-beta 1 in adult T cell leukemia. J. Exp. Med. **172:**121–129.
30. **Letesson, J. J., A. Mager, M. Mammerickx, A. Burny, and A. Depelchin.** 1990. B cells from bovine leukemia virus- (BLV) infected sheep with hematological disorders express the CD5 T cell marker. Leukemia **4:**377–379.
31. **Letesson, J. J., A. Van den Broecke, Y. Marbaix Cleuter, M.**

Delcommenne, A. Mager, M. Mammerickx, A. Burny, and A. Depelchin. 1991. FACS analysis of bovine leukemia virus (BLV)-infected cell lines with monoclonal antibodies (mAbs) to B cells and to monocytes/macrophages. Vet. Immunol. Immunopathol. **27:**207–213.

32. **Levy, D., R. Kettmann, P. Marchand, S. Djilali, and A. L. Parodi.** 1987. Selective tropism of bovine leukemia virus (BLV) for surface immunoglobulin-bearing ovine lymphocytes. Leukemia **1:**463–465.
33. **Lewin, H. A.** 1989. Disease resistance and immune response genes in cattle: strategies for their detection and evidence of their existence. J. Dairy Sci. **72:**1334–1348.
34. **Lilienbaum, A., M. Duc Dodon, C. Alexandre, L. Gazzolo, and D. Paulin.** 1990. Effect of human T-cell leukemia virus type I Tax protein on activation of the human vimentin gene. J. Virol. **64:**256–263.

34a. **MacHugh, N.** Unpublished data.

35. **Mammerickx, M., R. Palm, D. Portetelle, and A. Burny.** 1988. Experimental transmission of leukosis in sheep: latency period of the tumoral disease. Leukemia **2:**103–107.
36. **McGuire, K. L., V. E. Curtiss, E. L. Larson, and W. A. Haseltine.** 1993. Influence of human T-cell leukemia virus type I *tax* and *rex* on interleukin-2 gene expression. J. Virol. **67:**1590–1599.
37. **McGuire, T. C., T. B. Crawford, and J. B. Henson.** 1971. Immunofluorescent localization of equine infectious anemia virus in tissue. Am. J. Pathol. **63:**283–294.
38. **Naessens, J., and D. J. Williams.** 1992. Characterization and measurement of CD5+ B cells in normal and Trypanosoma congolense-infected cattle. Eur. J. Immunol. **22:**1713–1718.
39. **Narayan, O., S. Kennedy-Stoskopf, D. Sheffer, D. E. Griffin, and J. E. Clements.** 1983. Activation of caprine arthritis-encephalitis virus expression during maturation of monocytes to macrophages. Infect. Immun. **41:**67–73.
40. **Nimer, S. D., J. C. Gasson, K. Hu, I. Smalberg, J. L. Williams, I. S. Chen, and J. D. Rosenblatt.** 1989. Activation of the GM-CSF promoter by HTLV-I and -II Tax proteins. Oncogene **4:**671–676.
41. **Richardson, J. H., A. J. Edwards, J. K. Cruickshank, P. Rudge, and A. G. Dalgleish.** 1990. In vivo cellular tropism of human T-cell leukemia virus type 1. J. Virol. **64:**5682–5687.
42. **Stott, M. L., M. C. Thurmond, S. J. Dunn, B. I. Osburn, and J. L. Stott.** 1991. Integrated bovine leukosis proviral DNA in T helper and T cytotoxic/suppressor lymphocytes. J. Gen. Virol. **72:**307–315.
43. **Willems, L., C. Bruck, D. Portetelle, A. Burny, and R. Kettmann.** 1987. Expression of cDNA clone corresponding to the long open reading frame (XBL-I) of the bovine leukemia virus. Virology **160:**55–59.
44. **Willems, L., H. Heremans, G. Chen, D. Portetelle, A. Billiau, A. Burny, and R. Kettmann.** 1990. Cooperation between bovine leukaemia virus transactivator protein and Ha-ras oncogene product in cellular transformation. EMBO J. **9:**1577–1581.
45. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislips, and I. S. Y. Chen.** 1990. HIV entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell **61:**213–222.