JOURNAL OF VIROLOGY, Apr. 1996, p. 2178–2183 Vol. 70, No. 4
0022-538X/96/$04.00+0

The Prevalence of Proviral Bovine Leukemia Virus in Peripheral Blood Mononuclear Cells at Two Subclinical Stages of Infection

MICHAEL L. MIRSKY,[1] COLLEEN A. OLMSTEAD,[2] YANG DA,[2] AND HARRIS A. LEWIN[2]*

Department of Veterinary Pathobiology[1] and Laboratory of Immunogenetics, Department of Animal Sciences,[2] University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Received 21 August 1995/Accepted 14 December 1995

The bovine leukemia virus (BLV) is an oncogenic retrovirus that is associated with the development of persistent lymphocytosis (PL) and lymphoma in cattle. While B lymphocytes have been shown to be the primary cellular target of BLV, recent studies suggest that some T lymphocytes and monocytes may be infected by the virus. Because virally altered functions of monocytes and/or T cells could contribute to the development of lymphoproliferative disease, we sought to clarify the distribution of the BLV provirus in subpopulations of peripheral blood mononuclear cells in seropositive cows with and without PL. $CD2^+$ T cells, monocytes, and $CD5^+$ and $CD5^-$ B cells were sorted by flow cytometry and tested for the presence of BLV by single-cell PCR. We did not obtain convincing evidence that peripheral blood monocytes or T lymphocytes contain the BLV provirus in seropositive cows with or without PL. In seropositive cows without PL ($n = 14$), BLV-infected $CD5^+$ and $CD5^-$ B cells accounted for 9.2% ± 19% and 0.1% ± 1.8% of circulating B lymphocytes, respectively. In cows with PL ($n = 5$), BLV-infected $CD5^+$ and $CD5^-$ B cells accounted for 66% ± 4.8% and 13.9% ± 6.6% of circulating B lymphocytes, respectively. The increase in lymphocyte numbers in cows with PL was entirely attributable to the 45-fold and 99-fold expansions of infected $CD5^+$ and $CD5^-$ B-cell populations, respectively. Our results demonstrate that B cells are the only mononuclear cells in peripheral blood that are significantly infected with BLV. On the basis of the absolute numbers of infected cells in seropositive, hematologically normal animals, there appear to be differences in susceptibility to viral spread in vivo that may be under the genetic control of the host.

The bovine leukemia virus (BLV) is a retrovirus that is closely related to the human T-cell leukemia viruses (HTLV-1 and HTLV-2) and the simian T-cell leukemia virus (13). These viruses are exogenous, have similar genomic organizations (42, 45), lack transforming oncogenes (8, 24, 46), integrate into dispersed sites within the host genome (23, 44, 55), and are restricted in their expression in vivo (reviewed in references 3 and 4). In addition to *gag*, *pol*, and *env* genes, viruses of the HTLV-BLV group possess a region 3′ to the *env* gene (42, 45) that encodes at least three other proteins that regulate the viral life cycle and are thought to contribute to pathogenesis in the host (22, 47).

The primary cellular target of BLV is the B lymphocyte (20, 39). Following the establishment of BLV infection, the host mounts a persistent antibody response to viral proteins, and virions can be isolated from cultured leukocytes of infected animals (11, 35). Although BLV infection remains subclinical in the majority of cattle, about one-third of BLV-infected cattle develop persistent lymphocytosis (PL) (11, 12), a polyclonal expansion of B lymphocytes (23). A smaller number of BLV-infected cattle (1 to 5%) develop oligo- or monoclonal lymphosarcoma with or without prior PL (11, 12, 21). The onset of PL usually occurs before the age of 5 years, whereas the peak incidence of lymphoma is between 5 and 8 years of age (11). The BLV provirus has been found in both $CD5^+$ and $CD5^-$ B-cell subsets of BLV-infected cows, and infection of $CD5^+$ cells was found to predominate in one cow with PL (43).

The susceptibility of cells other than B lymphocytes to BLV infection in vivo is less clear because of the relative magnitude of B-cell infection and problems in obtaining highly purified cell populations. Monocytes were first implicated as potential carriers of BLV in sheep on the basis of cell morphology and in situ hybridization (26). Heeney et al. presented evidence that BLV was present in 5 to 40% of adherence-purified monocytes but not in T cells or granulocytes from peripheral blood of BLV-infected cows with or without PL (17). Evidence that T cells are targets for BLV infection has been obtained by others through immunoaffinity depletion of B-cells and monocytes from peripheral blood (54), expansion of T cells in culture with interleukin 2 (50), or positive selection of T cells with immunomagnetic beads (43).

In the present study, we sought to clarify the cellular distribution of BLV and to determine the prevalence of the provirus in peripheral blood leukocyte subpopulations of naturally infected cows with and without PL. For this purpose we isolated $CD5^+$ and $CD5^-$ B cells, monocytes, and T cells from peripheral blood by flow cytometry and tested them by PCR for the presence of the BLV provirus with a recently developed single-cell methodology (37). The results of these experiments indicate that the provirus may be limited to B cells in peripheral blood of infected cows with and without PL and that PL represents expansion of the infected $CD5^+$ and $CD5^-$ B-cell subsets.

MATERIALS AND METHODS

Animals. A total of 19 BLV-infected Holstein-Friesian cows, 14 without PL and 5 with PL, were selected for study. The cows were maintained within the University of Illinois dairy research herd, and all were naturally infected with BLV. Since 1985, the animals in this herd have been tested annually for BLV infection by an agar gel immunodiffusion test with a commercially prepared antigen (Leukassay B; Pitman Moore, Atlanta, Ga.) according to the manufacturer's specifications. The seroprevalence of BLV in the herd is 85% for cows over 3 years of age, and the prevalence of PL is about 20% (29). Total leukocyte and differential counts were determined by standard hematological techniques.

* Corresponding author. Mailing address: Laboratory of Immunogenetics, Department of Animal Sciences, University of Illinois at Urbana-Champaign, 206 Edward R. Madigan Laboratory, Urbana, IL 61801. Phone: (217) 333-5998. Fax: (217) 244-5617.

Cows were classified as lymphocytotic by a standard leukosis key for cattle (31); animals diagnosed with PL were lymphocytotic in two consecutive tests at least 3 months apart. The cows used in this study ranged in age from 41 to 100 months and had seroconverted to BLV 6 to 68 months prior to testing. Cows with PL were, on average, 15 months older and had seroconverted 22 months earlier than cows without PL.

Cell separation. Blood was collected by jugular venipuncture into acid citrate-dextrose (Vacutainer; Becton Dickinson Co., Rutherford, N.J.) and maintained at room temperature prior to cell separation. The buffy coat was harvested following centrifugation at 1,000 × *g* for 10 min at room temperature and diluted 1:1 in cell separation medium (phosphate-buffered saline [PBS] containing 0.2% sodium azide, 0.5% bovine serum albumin [Sigma, St. Louis, Mo.], and 2 mM EDTA). The diluted cells were layered over Histopaque (specific gravity, 1.077; Sigma) in 15-ml conical tubes and centrifuged at 1,000 × *g* for 20 min at room temperature. Peripheral blood mononuclear cells (PBMC) were removed from the gradient interface and washed in cold cell separation medium, once at 700 × *g* for 10 min at 4°C and then twice at 400 × *g* for 12 min at 4°C, to remove platelets. Cells were resuspended in cold PBS containing 0.2% sodium azide (PBSN), counted on a hemacytometer, assessed for viability with a phase-contrast microscope, and adjusted to 4×10^6 to 6×10^6 viable cells per ml.

Antibodies. The monoclonal antibodies used in this study were B26A (anti-bovine CD2; 5 μg/ml), Cact61A (anti-bovine γ/δ T-cell receptor; 6.67 μg/ml), and B29A (anti-bovine CD5; 6.67 μg/ml), all purchased from VMRD (Pullman, Wash.). The IL-A109 monoclonal antibody (anti-monocyte/macrophage) (16), a 1:500 dilution of unpurified ascites, was kindly supplied by N. MacHugh, International Laboratory for Research on Animal Diseases, Nairobi, Kenya. B lymphocytes were labeled directly with fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of rabbit anti-bovine immunoglobulin G (IgG) (heavy and light chain specific; Accurate Chemical, Westbury, N.Y.). Phycoerythrin-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG or IgM (Jackson Immunoresearch, West Grove, Pa.) were used for indirect labeling of PBMC populations. Murine IgG2a and IgM (Southern Biotechnology, Birmingham, Ala.) were used as irrelevant-isotype controls for indirect immunofluorescence.

Immunophenotyping. Cell populations were analyzed and sorted by dual-color immunofluorescence. Prior to antibody labeling, aliquots of 2×10^6 to 3×10^6 PBMC were incubated at 4°C for 10 min in 100 μl (3 mg of protein per ml) of normal goat serum (Jackson Immunoresearch) to block Fc receptors. After the goat serum was removed, the cells were resuspended in 100 μl of the appropriate monoclonal antibody, incubated for 40 min at 4°C, and then washed twice in 1 ml of PBSN. Cells labeled with IL-A109, B26A, or Cact61A were then resuspended in 100 μl of a cocktail containing phycoerythrin-conjugated goat anti-mouse IgM and the fluorescein isothiocyanate-conjugated rabbit anti-bovine IgG. Cells labeled with B29A were resuspended in 100 μl of phycoerythrin-conjugated goat anti-mouse IgG. Cells were then incubated for 40 min on ice in the dark and washed twice in 1 ml of PBSN. Cells labeled with B29A were resuspended in 100 μl of fluorescein isothiocyanate-conjugated rabbit anti-bovine IgG, incubated for 40 min on ice in the dark, and then washed as described above. Controls for each animal were prepared identically and included cells labeled with isotype-matched irrelevant antibodies and cells to which specific primary antibodies were not added.

Cell analysis and sorting. Cell sorting and analysis were performed as described previously (37), with modifications, with a flow cytometer equipped with a 5-W argon ion laser and a single-cell deposition device (EPICS 751 with Autoclone; Coulter Corp., Miami, Fla.) with a Cicero data acquisition and sorting system (Cytomation, Inc., Fort Collins, Colo.). Cell populations were analyzed with Cyclops software (Cytomation, Inc.) on the basis of light scatter and log green fluorescence versus log red fluorescence. At least 5,000 events were collected in list mode with a gate set to include all live cells on the basis of forward light scatter versus 90° light scatter. $CD5^+$ B cells were selected as $B29A^+$ and surface immunoglobulin positive (sIg^+), $CD5^-$ B cells were selected as $B29A^-$ sIg^+, $CD2^+$ T cells were selected as $B26A^+$ sIg^-, γ/δ T cells were selected as $Cact61A^+$ sIg^-, and monocytes were selected as $IL\text{-}A109^+$ sIg^-. Absolute numbers of each cell population analyzed by flow cytometry were determined by the following formula: absolute cell number = total leukocyte count × percent $PBMC_{differential}$ × percent fluorescent cells. Total B cells were calculated as the sum of $CD5^+$ sIg^+ and $CD5^-$ sIg^+ cells. Subpopulations of cells were selected for sorting by using the four-quadrant dual-fluorescence analysis histogram.

To each well of 96-well plates (M J Research, Watertown, Mass.), 10 μl of lysis solution (100 mM KOH, 25 mM dithiothreitol) was added. Each cell population was sorted into a separate 96-well plate at a flow rate of 350 to 550 cells per s as follows: 24 wells received 1,000 cells, 32 wells received 100 cells, and 24 wells received 10 cells. When percentages of infected cells were greater than 10%, animals were retested with 1, 10, and/or 20 cells per well. Each plate also included at least six wells to which no cells were added (negative controls) and two wells to which 0.01 ng of BL3* DNA (see below) was added (positive controls). After sorting was completed, the plates were incubated in a closed, humidified plastic chamber for 20 min at 65°C. Reactions were neutralized with 10 μl of a solution containing 450 mM Tris-HCl (pH 8.4), 150 mM KCl, and 100 mM HCl, and plates were stored at −20°C until used.

To determine the accuracy of cell sorting, at least 10^5 cells from one cow with PL and the same quantity of cells from one cow without PL were labeled with anti-bovine CD2 or anti-monocyte/macrophage MAb and sorted into 1.5-ml Eppendorf tubes containing 0.5 ml of PBS at 4°C. The collected cells were reanalyzed by flow cytometry, and purity was determined as the percentage of viable cells falling within the originally gated quadrant. In these experiments, ≥92% of the reanalyzed cells were specifically labeled. On the basis of this result, the overall efficiency of sorting T cells and monocytes was conservatively estimated at 90%. Because cell sorting was less than 100% specific and B cells were the predominant cell population containing provirus (see below), the number of positive PCRs due to contaminating B cells was estimated for T-cell and monocyte populations as follows: expected number of B-cell-contaminated reactions = percent B cells × percent infected B cells × (1 − sorting efficiency) × number of cells per well × number of wells tested. To distinguish whether the observed infection of $CD5^-$ B cells was attributable to contaminating $CD5^+$ B cells, the same formula was applied, substituting $CD5^+$ B cells for B cells.

PCR coamplification of cellular prolactin and BLV envelope sequences. The amplification of viral and cellular sequences was performed with heminested primers in two stages as described previously (37) with some modifications. The detection system has previously been shown to be capable of detecting a single copy of a gene (28, 37). Briefly, primers for amplifying a 177-bp fragment of the cellular prolactin gene (PRL) (28) and a 210-bp fragment of the BLV envelope gene (ENV) (37) were added to each well, and the wells were covered with 20 μl of liquid wax (ChillOut-14; M J Research). Reaction mixtures were heated to 94°C in a 96-well thermal cycler (PTC-100; M J Research) for 4 min and then held at 92°C while a potassium-free PCR mixture was added through the oil to each well, providing a final concentration of 50 mM KCl, 100 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 100 μM (each) deoxynucleoside triphosphate (U.S. Biochemical, Cleveland, Ohio), 1 U of *Taq* polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, Conn.), 0.2 μM (each) PRL primer, and 0.4 μM (each) BLV ENV primer in a final volume of 50 μl. The cycling parameters used in the first PCR were 10 cycles of 1 min at 92°C, 3 min at 52°C, and 1 min at 72°C; 10 cycles of 1 min at 92°C, 2 min at 55°C, and 1 min at 72°C; 20 cycles of 1 min at 92°C, 1 min at 57°C, and 1 min at 72°C; and 4 min at 72°C. Aliquots (2 μl) of the first-round PCR product were transferred to each of two 50-μl second-round amplification mixtures containing heminested PRL or BLV ENV primers (37). The cycling parameters for the second round of amplification were 30 cycles of 1 min at 92°C and 1 min at 58°C, followed by a 5-min final extension at 72°C. Products of the two second-round PCRs from each initial well were combined and visualized with UV light after electrophoresis in 6% polyacrylamide gels and staining with ethidium bromide. Restriction enzyme digestion of the second-round BLV ENV PCR product with *Hae*III (Bethesda Research Laboratories, Gaithersburg, Md.) was performed as previously described (37) to verify the identity of this product.

Calculation of frequencies of infected cells. DNA was isolated by the protocol of Miller et al. (36). The BLV-infected and uninfected B-lymphoblastoid cell lines BL3* and $BL3^0$ (41) were used to establish the operational sensitivity for simultaneous detection of both viral and cellular templates as described previously (37). In preliminary experiments, the operational sensitivity for detecting BLV ENV was 1.00, 0.99, 0.91, 0.86, or 0.89 when a single infected BL3* cell was sorted into wells containing 0, 1, 10, 100, or 1,000 uninfected cells, respectively. BLV ENV was not identified in any tests in the absence of the PRL product. Neither BLV ENV nor PRL was detected in negative-control reactions.

A computer program developed for single-cell analysis (37) was used to estimate the frequency of infection in each cell population. Variables used by the program included the operational sensitivity, the number of wells containing PRL alone and the number containing PRL plus BLV ENV, the number of cells tested per well, and the number of wells examined. An adjustment for DNA contamination was not necessary because negative-control wells showed no evidence of contamination. Absolute numbers of infected cells in each population were calculated as the absolute number times the frequency of infected cells.

Statistical analysis. Percentages and absolute numbers for total, infected, and uninfected leukocyte subpopulations were tested for normality within each group of animals by a univariate procedure (SAS/STAT, 1990; SAS Institute, Cary, N.C.). Curvilinear regression was used when necessary to transform nonnormal data. For animals with or without PL, the relationships among numbers and percentages of circulating mononuclear leukocytes, infection rates, animal age, and months of infection were determined by regression analysis with a general linear-models procedure (SAS/STAT, 1990). When one of these parameters was significantly influenced by age or by months of infection, it was included as a covariable for comparisons between disease groups. For each variable, differences between cows with PL and those without PL were tested for significance by using a two-sample *t*-test procedure for unequal sample sizes (SAS/STAT, 1990). When necessary, the approximate *t*-test statistic was used for comparison of samples having unequal variance by Satterthwaite's approximation for degrees of freedom.

RESULTS

Effects of age, months of infection, and PL status on total leukocyte and PBMC subpopulations. In cows without PL, total leukocyte, lymphocyte, and B-cell counts declined with increasing animal age ($P < 0.05$ for all comparisons) whereas

TABLE 1. PBMC subpopulations in BLV-infected cows with and without PL

Cell type	Cows without PL[a] (n = 14)			Cows with PL (n = 5)		
	% of PBMC (mean ± SD)	No. of PBMC/mm^3		% of PBMC (mean ± SD)	No. of PBMC/mm^3	
		Count	Range		Count	Range
CD5$^+$ B cells	17.8 ± 7.9***	622 ± 346**	211–1,320	52.3 ± 5.1	6,504 ± 2,082	3,740–8,946
CD5$^-$ B cells	11.1 ± 4.7	401 ± 195*	129–691	14.3 ± 6.2	1,779 ± 967	941–2,841
CD2$^+$ T cells	40.9 ± 11.8***	1,441 ± 425**	809–2,333	19.0 ± 3.7	2,292 ± 598	1,658–3,021
γ/δ T cells[b]	4.5 ± 1.8	152 ± 46**	102–217	2.6 ± 0.1	279 ± 72	205–376
Monocytes	21.5 ± 6.4***	790 ± 380	405–1,786	7.1 ± 1.2	861 ± 243	634–1,208

[a] *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$. Comparisons are between cows without PL and cows with PL.
[b] n = 9 for cows without PL; n = 4 for cows with PL.

the percentage of γ/δ T cells increased ($P < 0.01$). Within the group of cows without PL, months of infection did not influence percentages or absolute numbers of the PBMC subpopulations. In cows with PL, percentages and absolute numbers of leukocytes and PBMC subpopulations were not influenced by age but the percentage of circulating monocytes increased with total time of infection ($P < 0.01$).

B lymphocytes accounted for 28.9 and 66.6% of PBMC in cows with and without PL, respectively (Table 1); the CD5$^+$ subset comprised the largest proportion of B cells in cows with (78.5%) and without (61.6%) PL. Cows with PL had ~10-fold and ~4-fold increases in absolute numbers of CD5$^+$ ($P < 0.01$) and CD5$^-$ ($P < 0.05$) subsets, respectively, and greater numbers of circulating CD2$^+$ (predominantly α/β [6]) and γ/δ T cells ($P < 0.01$) compared with cows without PL (Table 1). Absolute numbers of monocytes did not differ significantly. The percentages of infected CD5$^+$ and CD5$^-$ B cells were strongly correlated among cows without PL but not among those with PL (Fig. 1).

BLV infection of CD5$^+$ and CD5$^-$ B cells. Of the 14 cows without PL tested, three had BLV in the CD5$^+$ B-cell subset only whereas 10 animals had BLV in both CD5$^+$ and CD5$^-$ B cells; provirus was not detected in B cells of one seropositive cow without PL (data not shown). Among the 10 cows without PL but with BLV-infected CD5$^-$ B cells, BLV ENV was observed at a frequency that was at least two to five times greater than the rate expected from CD5$^+$ B-cell contamination (see Materials and Methods; data not shown). Among all cows without PL, BLV-infected CD5$^+$ and CD5$^-$ B cells accounted for means (± standard deviations) of 9.2% ± 19.0% and 0.9% ± 1.8% of peripheral blood B cells, respectively (Table 2). Of these animals, 3 had >500 infected cells per mm^3 while the remaining 11 had ≤20 infected cells per mm^3 (Fig. 2). Among cows without PL, there was a tendency (P <0.10) for lymphocyte numbers to increase as the percentage of infected CD5$^+$ B cells increased (Fig. 2).

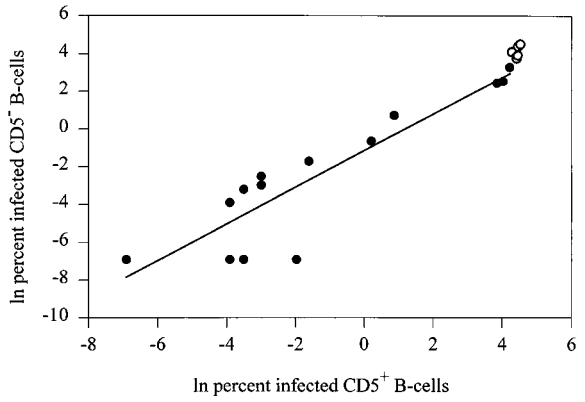

FIG. 1. Relationship between percentages of BLV-infected CD5$^+$ and CD5$^-$ B cells in cows with and without PL. The percentage of infected CD5$^-$ B cells (y) for cows without PL (●) was $-1.30 - 0.97x$ ($r^2 = 0.81$; $P < 0.0001$), and for cows with PL (○) it was $-0.22 - 1.46x$ ($r^2 = 0.18$; $P > 0.05$).

Among cows with PL, 66.0% ± 4.8% of CD5$^+$ B cells were BLV infected whereas 13.9% ± 6.6% of CD5$^-$ B cells were infected. The absolute number of uninfected B cells declined with age and with increasing time of infection (P <0.05 for both comparisons; data not shown). Increases in absolute numbers of infected B cells in cows with PL reflected 45-fold (P <0.0001) and 99-fold (P <0.0001) increases in the CD5$^+$ and CD5$^-$ subsets, respectively, compared with cows without PL, whereas absolute numbers of uninfected CD5$^+$ and CD5$^-$ B cells were not significantly different (Table 2).

BLV infection of T lymphocytes and monocytes. BLV was detected in sorted populations of CD2$^+$ (predominantly α/β) T cells and monocytes from cows with and without PL (Table 2). The estimated infection rates were compared with expected rates of B-cell contamination in the sorted cells. For only one cow without PL were the estimated infection rates of monocytes and CD2$^+$ T cells slightly higher than the estimated B-cell contamination rate; in all other cows tested, the infection rates in these cell populations were lower than B-cell contamination rates (data not shown). Double sorting of T cells from a cow with PL markedly decreased the estimated infection rate in comparison with that of cells isolated by the standard one-time sorting procedure: of 36 wells receiving 100 cells each, 26 and 7 were BLV positive by single and double sorting, respectively, with infection rates of 1.4% ± 0.2% and 0.2% ± 0.1%, respectively. These results suggest that peripheral α/β T cells and monocytes are not a major reservoir for BLV provirus.

Sorted γ/δ T cells from one cow with PL were tested for BLV provirus. The estimated percentage of infected cells was similar to that observed for CD2$^+$ T cells in the same animal (data not shown). Results obtained for this animal suggest that peripheral γ/δ T cells are not a reservoir for BLV provirus. Because of the time required to sort large numbers of γ/δ T cells and our inability to completely exclude B-cell contamination in sorted γ/δ T cells, further study of the susceptibility of this population to BLV infection was not attempted.

DISCUSSION

In BLV-seronegative cows, lymphocyte and B-cell numbers decrease with age, thus providing a basis upon which various keys have been developed to identify cows at risk for develop-

TABLE 2. BLV-infected PBMC subpopulations in cows with and without PL

Cell type	Cows without PL[a] ($n = 14$)			Cows with PL ($n = 5$)		
	% of PBMC (mean ± SD)	No. of PBMC/mm^3		% of PBMC (mean ± SD)	No. of PBMC/mm^3	
		Count	Range		Count	Range
CD5$^+$ B cells[b]						
BLV infected	9.2 ± 19.0***	121 ± 244***	0–729	66.0 ± 4.8	5,467 ± 1,856	3,246–8,293
Uninfected	49.4 ± 17.8***	501 ± 289	211–1,320	12.7 ± 7.2	1,036 ± 699	494–2,248
CD5$^-$ B cells[b]						
BLV infected	0.9 ± 1.8***	12 ± 25***	0–87	13.9 ± 6.6	1,183 ± 843	538–2,546
Uninfected	40.5 ± 18.4***	389 ± 192	94–655	7.4 ± 5.6	596 ± 494	204–1,406
CD2$^+$ T cells (BLV infected)[c]	0.18 ± 0.36	3 ± 6	0–22	1.5 ± 0.8	32 ± 13	18–48
Monocytes (BLV infected)[c]	0.1 ± 0.2	1 ± 2	0–7	0.6 ± 0.5	8 ± 8	2–21

[a] ***, $P < 0.0001$. Comparisons are between cows without PL and cows with PL.
[b] Percentages are of total B-cell population.
[c] Percentages are of total subset population.

ing PL and lymphoma (29, 31, 32, 40). In contrast, lymphocyte and B-cell numbers increase in BLV-seropositive cows until about 5 years of age (29). Consistent with these earlier findings, in the present study, cows with PL had markedly greater numbers of PBMC and B cells than did BLV-infected cows without PL. The magnitude of the expansion of CD5$^+$ and CD5$^-$ B-cell subsets observed in animals with PL was similar to results reported previously (34). Among cows without PL, 3 of 14 animals had greater total numbers (though not percentages) of lymphocytes and CD5$^+$ B cells than did other cows in this population (data not shown), suggesting that absolute numbers of circulating CD5$^+$ B cells are predictive for susceptibility to the development of PL. Among cows with PL we observed a significant increase in the percentage of circulating monocytes relative to the length of infection, although this relationship was not matched by a correlated change in total numbers of monocytes. This could be indicative of the ability of infected cows to maintain constant numbers of monocytes in the face of B-cell dysregulation.

A highly sensitive and quantitative method for detecting BLV genomic sequences was used to more clearly establish the cell tropism of BLV in naturally infected cows at different stages of infection. Although the method has the ability to detect the replicative intermediate form of BLV, only provirus was detected in our experiments because BLV ENV is not expressed in infected peripheral blood cells in vivo (23). B lymphocytes were the primary, if not the exclusive, population infected by BLV. B cells containing the provirus accounted for 46 to 65% and 0 to 18% of the PBMC in BLV-seropositive cows with and without PL, respectively. Others who investigated the prevalence of BLV in leukocytes ex vivo have found lower (21, 23) or higher (10, 25) percentages of cells containing the provirus at these stages of infection. Such discrepancies may reflect differences in the sensitivities of the methods employed for quantitation of virus-infected cells. Among cows without PL which had low absolute numbers of BLV-infected B cells, provirus tended to be restricted to the CD5$^+$ B cells (data not shown). In contrast, although BLV was present in both B-cell subsets of the three cows without PL which had large numbers of circulating infected cells, 10-fold-greater numbers of CD5$^+$ B cells contained provirus (data not shown). Cows that progressed to PL were characterized by almost 50-fold and 100-fold expansions of infected CD5$^+$ and CD5$^-$ B cells, respectively, without a concomitant increase in absolute numbers of uninfected B cells (Table 2). These findings suggest that in animals susceptible to the development of PL, BLV may infect a relatively large and, thereafter, stable population of early or pre-B cells with the capacity for self-renewal and/or proliferation. PL could then develop through the expansion of the infected population(s) rather than the recruitment of newly infected clones. The constant percentages but increasing absolute numbers of infected cells in cows with PL (Fig. 2) also suggest that B-cell expansion is a result of proliferation rather than prolonged survival of infected cells. The bimodal distribution of BLV-infected cells among BLV-infected cows without PL observed in this and other studies (25) may reflect genetic influences on viral spread and disease progression in vivo (1, 5). We are currently investigating the genetic basis for resistance to viral spread in greater depth using a subset of the animals tested in the present study.

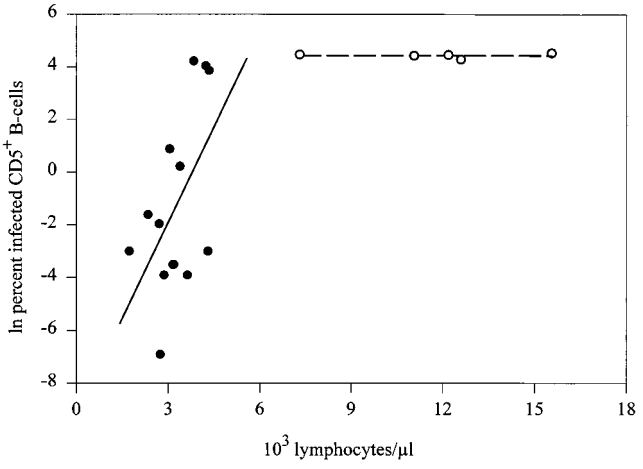

FIG. 2. Relationship between percentage of BLV-infected CD5$^+$ B cells and total lymphocyte numbers in cows with and without PL. The percentage of BLV-infected CD5$^+$ B cells (y) for cows without PL (●) was $-13.7 + 0.002x$ ($r^2 = 0.29$; $P < 0.10$), and for cows with PL (○) it was $-0.21 + 3.4 \times 10^{-6}x$ ($r^2 = 0.01$; $P > 0.10$).

The apparent expansion of the CD5$^+$ B-cell subset with progression to PL has important implications for understanding the pathogenesis of disease in BLV-infected cattle. In mice, B cells expressing CD5 are thought to be a unique, self-per-

petuating lineage (reviewed in reference 19). In contrast, the expression of CD5 by human B cells appears to be indicative of B-cell activation (reviewed in reference 30). It was suggested that CD5 expression in cows with PL might be induced by viral *tax*-mediated *trans*-activation (7), but the number of circulating cells expressing viral message in animals with PL is inadequate to support this theory (14a, 15, 23). Others have suggested that BLV selectively infects and/or induces expansion of the $CD5^+$ B-cell subset (34). In the present study, we have shown that $CD5^-$ B cells also contain BLV provirus in cows with and without PL; furthermore, marked expansion of the $CD5^-$ B-cell subset was observed with the development of PL. It is possible that B-cell progenitors expressing CD5 are more susceptible to BLV infection, which could account for the greater proportion of $CD5^+$ B cells containing the provirus as infection progresses.

Currently we favor the view that CD5 is a marker of cellular activation in cattle. Cows infected with BLV have increased numbers of actively cycling cells among PBMC (33). T-cell-depleted PBMC from cows with PL are responsive to interleukin 2 (33), and increased numbers of B cells can be induced to express the interleukin 2 surface receptor (49). In addition, we (unpublished data) and others (49) have observed fluctuations in the numbers of cells expressing CD5 in cows without PL and in non-BLV-infected cows. If CD5 proves to be an activation marker of bovine B cells, it is likely that infected precursors generate both $CD5^+$ and $CD5^-$ cells. The expression of CD5 by BLV-infected B cells may result directly from proviral integration and/or cytokines in the lymphoid microenvironment. BLV-infected $CD5^-$ B cells might include cells in the G_0 state, the pre-$CD5^+$ pool, dying cells with decreased CD5 expression, or pretransformed B cells.

As a group, cows with PL had greater numbers of both α/β ($CD2^+$) and γ/δ T cells than did cows without PL. In comparison with BLV-seronegative cows, absolute numbers of T cells have been reported to increase (49, 53), decrease (14, 48), or remain normal (51) in cows with PL, whereas seropositive cows without PL were reported to have increased (53) or decreased (14, 51) numbers of T cells. Differences among studies have been attributed to alterations in the immune system relative to the duration of infection (27, 48, 51). Recently, we also have observed that absolute T-cell numbers tend to increase with duration of infection in some cows that are genetically susceptible to the development of PL (36a). Furthermore, age-dependent differences in the composition of the bovine peripheral blood lymphocyte population (29, 31) may influence the interpretation of results. Unfortunately, independent data regarding absolute numbers of γ/δ T cells in peripheral blood of BLV-infected cows are not currently available. As the focus of this study was to determine the cellular distribution of BLV provirus in infected cattle, uninfected animals were not included in the experimental design. Studies currently under way in our laboratory will provide the information necessary to interpret the observed differences in α/β and γ/δ T-cell numbers between cows with PL and those without PL.

Monocytes and T cells have been implicated as targets of BLV infection in vivo (17, 43, 50, 54). In the present study, the highest levels of BLV infection detected in sorted monocytes (1.5%) and T cells (2.8%) were observed in cows with PL. However, double sorting of cells for reanalysis and/or PCR testing established that significant proportions of the positive reactions were due to contamination with infected B cells during sorting. Thus, we were unable to determine whether circulating T cells or monocytes can be infected, but it is unlikely that >1% of either of these populations harbors the provirus. Our inability to detect BLV provirus in significant numbers of circulating T cells and monocytes may have been due to the sequestration of these cells within lymphoid or other tissues. Studies of human immunodeficiency virus type 1 have demonstrated that the viral burden is far greater in lymphoid tissues than it is in peripheral blood (9, 38) and that viral expression is concentrated in areas of rapid cell turnover (18, 52). Expression of BLV has been detected in lymph nodes (17) and mammary epithelial cells (2), but the number of infected cells in these locations has not been established. While it is possible that BLV is cytolytic for T cells or monocytes in vivo, we did not observe that disease progression was associated with a decline in absolute numbers of either population. On the basis of these results, we believe that infected peripheral T cells, if present, do not play an important role in BLV pathogenesis.

Our results illustrate the power and utility of single-cell PCR methodology for monitoring the clinical progression of retrovirus-associated disease. The integration of flow cytometry and PCR allows for rapid selection of phenotypically defined cell populations, direct testing of selected populations, and reliable quantitation of cells containing viral (or cellular) sequences of interest. We have been able to clearly identify BLV-infected cells even when they are present at very low frequencies in the circulation and to identify animals in which infection has spread without other evidence of disease progression. By applying a quantitative single-cell PCR methodology to the study of cell populations in the spleens, lymph nodes, and bone marrow of infected cows, we hope to clarify the distribution of the virus in vivo and monitor changes in infected cell populations both at various stages of infection and as a function of host major histocompatibility complex genotype.

ACKNOWLEDGMENTS

We thank Julie Auger, Gary Durack, and Karen Magin of the University of Illinois Flow Cytometry Facility for technical support, Suzanne Voss for technical assistance, and Gene McCoy for help in obtaining and bleeding cattle for this study.

This project was supported in part by the NIH NCI (RO1CA59148) and the U.S. Department of Agriculture, National Research Initiative (92-37204-7956).

REFERENCES

1. **Abt, D. A., R. R. Marshak, J. F. Ferrer, C. E. Piper, and D. M. Bhatt.** 1976. Studies on the development of persistent lymphocytosis and infection with the bovine C-type leukemia virus (BLV) in cattle. Vet. Microbiol. **1:**287–300.
2. **Buehring, G. C., P. M. Kramme, and R. D. Schultz.** 1994. Evidence for bovine leukemia virus in mammary epithelial cells of infected cows. Lab. Invest. **71:**359–365.
3. **Burny, A., Y. Cleuter, R. Kettmann, M. Mammerickx, G. Marbaix, D. Portetelle, A. van den Broecke, 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.
4. **Cann, A. J., and I. S. Y. Chen.** 1990. Human T-cell leukemia virus types I and II, p. 1501–1527. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsh, J. L. Melnick, T. P. Monath, and B. R. Roizman (ed.), Virology, 2nd ed. Raven Press, Ltd., New York.
5. **Croshaw, J. E., D. A. Abt, R. R. Marshak, W. C. D. Hare, and J. Switzer.** 1963. Pedigree studies in bovine lymphosarcoma. Ann. N.Y. Acad. Sci. **108:**1193–1202.
6. **Davis, W. C., M. J. Hamilton, Y.-H. Park, R. A. Larsen, and C. R. Wyatt.** 1990. Ruminant leukocyte differentiation molecules, p. 47–70. *In* O. Barta (ed.), MHC, differentiation antigens, and cytokines in animals and birds. Bar-Lab, Blacksburg, Va.
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. **Deschamps, J., R. Kettmann, and A. Burny.** 1981. Experiments with cloned complete tumor-derived bovine leukemia virus information prove that the virus is totally exogenous to its target animal species. J. Virol. **40:**605–609.
9. **Embretson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, and A. T. Haase.** 1993. Massive covert infection of helper T lymphocytes and

macrophages by HIV during the incubation period of AIDS. Nature (London) **362:**359–362.
10. **Esteban, E. N., R. M. Thorn, and J. F. Ferrer.** 1985. Characterization of the blood lymphocyte population in cattle infected with the bovine leukemia virus. Cancer Res. **45:**3225–3230.
11. **Ferrer, J. F.** 1980. Bovine lymphosarcoma. Adv. Vet. Sci. Comp. Med. **24:**1–68.
12. **Ferrer, J. F., R. R. Marshak, D. A. Abt, and S. J. Kenyon.** 1978. Persistent lymphocytosis in cattle: its cause, nature and relation to lymphosarcoma. Ann. Rech. Vet. **9:**851–857.
13. **Francki, R. I. B., C. M. Fauquet, D. L. Knudson, and F. Brown (ed.).** 1991. Classification and nomenclature of viruses. Fifth report of the International Committee on Taxonomy of Viruses. Arch. Virol. **1991**(Suppl. 2)**:**290–299.
14. **Gatei, M. H., R. B. Brandon, H. M. Naif, M. W. McLennan, R. C. W. Daniel, and M. F. Lavin.** 1989. Changes in B cell and T cell subsets in bovine leukaemia virus-infected cattle. Vet. Immunol. Immunopathol. **23:**139–147.
14a. **Gaynor, E. M., M. L. Mirsky, and H. A. Lewin.** Use of flow cytometry and RT-PCR for detecting gene expression by single cells. BioTechniques, in press.
15. **Haas, L., T. Divers, and J. W. Casey.** 1992. Bovine leukemia virus gene expression in vivo. J. Virol. **66:**6223–6225.
16. **Hall, G. A., P. Sopp, and C. J. Howard.** 1993. An investigation of temporary workshop clusters reacting with cells of the mononuclear phagocytic system. Vet. Immunol. Immunopathol. **39:**225–236.
17. **Heeney, 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.
18. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature (London) **373:**123–126.
19. **Kantor, A. B.** 1991. The development and repertoire of B-1 cells (CD5 B cells). Immunol. Today **12:**389–391.
20. **Kenyon, S. J., and C. E. Piper.** 1977. Cellular basis of persistent lymphocytosis in cattle infected with bovine leukemia virus. Infect. Immun. **16:**891–897.
21. **Kettmann, R., Y. Cleuter, M. Mammerickx, M. Meunier-Rotival, G. Bernardi, A. Burny, and H. Chantrenne.** 1980. Genomic integration of bovine leukemia provirus: comparison of persistent lymphocytosis with lymph node tumor form of enzootic bovine leukosis. Proc. Natl. Acad. Sci. USA **77:**2577–2581.
22. **Kettmann, R., D. Couez, D. Grégoire, and A. Burny.** 1987. Leukemogenesis by bovine leukemia virus, p. 153–160. *In* A. Burny and M. Mammerickx (ed.), Enzootic bovine leukosis and bovine leukemia virus. Martinus Nijhoff, Boston.
23. **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.
24. **Kettmann, R., D. Portetelle, M. Mammerickx, Y. Cleuter, D. Dekegel, M. Galoux, J. Ghysdael, A. Burny, and H. Chantrenne.** 1976. Bovine leukemia virus: an exogenous RNA oncogenic virus. Proc. Natl. Acad. Sci. USA **73:**1014–1018.
25. **Kramme, P. M., C. B. Thomas, and D. Schultz.** 1994. The contribution of bovine leukaemia virus infected B-cells to the number of circulating B-cells in cattle. Comp. Haematol. Int. **4:**96–101.
26. **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.
27. **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.
28. **Lewin, H. A., K. Schmitt, R. Hubert, M. J. T. van Eijk, and N. Arnheim.** 1992. Close linkage between bovine prolactin and BoLA-DRB3 genes: genetic mapping in cattle by single sperm typing. Genomics **13:**44–48.
29. **Lewin, H. A., M.-C. Wu, T. J. Nolan, and J. A. Stewart.** 1988. Peripheral B lymphocyte percentage as an indicator of subclinical progression of bovine leukemia virus infection. J. Dairy Sci. **71:**2526–2534.
30. **Lydyard, P. M., and P. Y. Youinou.** 1994. Human $CD5^+$ (B1) B cells in health and disease. Fundam. Clin. Immunol. **2:**9–25.
31. **Mammerickx, M., R. J. Lorenz, O. C. Straub, W. J. C. Donnelly, J. C. Flensburg, G. Gentile, L. M. Markson, A. A. Ressang, and S. M. Taylor.** 1978. Bovine hematology. III. Comparative breed studies on the leukocyte parameters of several European cattle breeds as determined in the common reference laboratory. Zentralbl. Veterinaer. Med. Reihe B **25:**257–267.
32. **Marshak, R. R., D. A. Abt, H. J. Bendixen, W. I. B. Beveridge, B. R. Burmester, D. Cohen, D. C. Dodd, R. M. Dutcher, W. C. D. Hare, W. F. H. Jarrett, J. E. Martin, V. Perman, G. Rosenberger, N. B. Shimkin, W. K. Silvers, J. W. Switzer, G. H. Theilen, and G. Winqvist.** 1968. Criteria for the determination of the normal and leukotic state in cattle. J. Natl. Cancer Inst. **41:**243–251.
33. **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.
34. **Meirom, R., J. Brenner, and Z. Trainin.** 1993. BLV-infected lymphocytes exhibit two patterns of expression as determined by Ig and CD5 markers. Vet. Immunol. Immunopathol. **36:**179–186.
35. **Miller, J. M., L. D. Miller, C. Olson, and K. G. Gillette.** 1969. Virus-like particles in phytohemagglutinin-stimulated lymphocyte cultures with reference to bovine lymphosarcoma. J. Natl. Cancer Inst. **43:**1297–1305.
36. **Miller, S. A., D. D. Dykes, and H. F. Polesky.** 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. **16:**1215.
36a. **Mirsky, M. L., et al.** Unpublished data.
37. **Mirsky, M. L., Y. Da, and H. A. Lewin.** 1993. Detection of bovine leukemia virus proviral DNA in individual cells. PCR Methods Appl. **2:**333–340.
38. **Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci.** 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature (London) **362:**355–358.
39. **Paul, P. S., K. A. Pomeroy, D. W. Johnson, C. C. Muscoplat, B. S. Handwerger, F. F. Soper, and D. K. Sorensen.** 1977. Evidence for the replication of bovine leukemia virus in the B lymphocytes. Am. J. Vet. Res. **38:**873–876.
40. **Perman, V., V. A. Dirks, and G. Fangmann.** 1970. Statistical evaluation of lymphocyte values on Minnesota dairy cattle. Am. J. Vet. Res. **31:**1217–1222.
41. **Romano, M. J., J. A. Stewart, and H. A. Lewin.** 1989. Phenotypic characterization of bovine lymphoblastoid cell lines. Vet. Immunol. Immunopathol. **23:**293–307.
42. **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.
43. **Schwartz, I., A. Bensaid, B. Polack, B. Perrin, M. Berthelemy, and D. Levy.** 1994. In vivo leukocyte tropism of bovine leukemia virus in sheep and cattle. J. Virol. **68:**4589–4596.
44. **Seiki, M., R. Eddy, T. B. Shows, and M. Yoshida.** 1984. Nonspecific integration of the HTLV provirus genome into adult T-cell leukaemia cells. Nature (London) **309:**640–642.
45. **Seiki, M., S. Hattori, Y. Hirayama, and M. Yoshida.** 1982. Human adult T-cell leukemia virus: molecular cloning of the provirus DNA and the unique terminal structure. Proc. Natl. Acad. Sci. USA **79:**6899–6902.
46. **Seiki, M., S. Hattori, Y. Hirayama, and M. Yoshida.** 1983. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. Proc. Natl. Acad. Sci. USA **80:**3618–3622.
47. **Smith, M. R., and W. C. Greene.** 1991. Molecular biology of the type I human T-cell leukemia virus (HTLV-I) and adult T-cell leukemia. J. Clin. Invest. **87:**761–766.
48. **Sordillo, L. M., C. R. Hicks, and G. M. Pighetti.** 1994. Altered interleukin-2 production by lymphocyte populations from bovine leukemia virus-infected cattle. Proc. Soc. Exp. Biol. Med. **207:**268–273.
49. **Stone, D. M., T. F. McElwain, and W. C. Davis.** 1994. Enhanced B-lymphocyte expression of IL-2R alpha associated with T lymphocytosis in BLV-infected persistently lymphocytotic cows. Leukemia **8:**1057–1061.
50. **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.
51. **Taylor, B. C., J. L. Stott, M. C. Thurmond, and J. P. Picanso.** 1992. Alteration in lymphocyte subpopulations in bovine leukosis virus-infected cattle. Vet. Immunol. Immunopathol. **31:**35–47.
52. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature (London) **373:**117–122.
53. **Williams, D. L., G. F. Amborski, and W. C. Davis.** 1988. Enumeration of T and B lymphocytes in bovine leukemia virus-infected cattle, using monoclonal antibodies. Am. J. Vet. Res. **49:**1098–1103.
54. **Williams, D. L., O. Barta, and G. F. Amborski.** 1988. Molecular studies of T-lymphocytes from cattle infected with bovine leukemia virus. Vet. Immunol. Immunopathol. **19:**307–323.
55. **Yoshida, M., S. Hattori, and M. Seiki.** 1985. Molecular biology of human T-cell leukemia virus associated with adult T-cell leukemia. Curr. Top. Microbiol. Immunol. **115:**157–175.