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Cellular Basis of Persistent Lymphocytosis in Cattle Infected with Bovine Leukemia Virus

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Peripheral blood lymphocytes from 14 cattle infected with the bovine leukemia virus (BLV) and 14 BLV-free cattle were examined by the membrane immunofluorescent antibody technique to detect surface immunoglobulin (S-Ig) and by the erythrocyte-antibody-complement (EAC) rosette test for the detection of complement receptors. Direct comparisons of the percentages of S-Ig-bearing cells and EAC rosette-forming cells in both infected and BLV-free animals showed no evidence for the presence of a substantial population bearing one surface marker but not the other. The data showed that cells with surface markers characteristic of B lymphocytes are responsible for most of the increase in peripheral blood lymphocytes which may accompany BLV infection. The release of infectious BLV and the spontaneous uptake of thymidine by short-term cultured peripheral blood lymphocytes from BLV-infected cattle were also studied. The results indicate that both of these activities are functions of B lymphocytes.

That persistent lymphocytosis (PL) may precede the development of clinically apparent bovine lymphosarcoma has long been recognized, but the nature of the relationship between PL and lymphosarcoma is unclear. Not all animals with lymphosarcoma have a history of PL (1) and many animals with PL never develop lymphosarcoma. Bovine leukemia virus (BLV) is associated with both PL and lymphosarcoma, but the in vitro cultivation of infected cells is necessary for the expression of the virus and its antigens (8, 12). BLV may be detected in short-term peripheral blood leukocyte cultures from cattle with histologically confirmed lymphosarcoma and from clinically normal cattle with or without PL (8, 12). Although the population of leukocytes which replicates BLV has not been identified, several authors have studied the cell surface markers of peripheral blood lymphocytes (PBL) of cattle with and without PL.

Muscoplat et al. (10) found that 28% of the lymphocytes in peripheral blood of normal cattle had surface immunoglobulins (S-Ig), whereas in the blood of cattle affected with PL, 63% of the lymphocytes bore S-Ig. Weiland and Straub found an average of 18% S-Ig-bearing cells in cattle without lymphocytosis and 67% in lymphocytotic animals (13).

E. C. Piper and J. F. Ferrer (Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, p. 109, M259), by using the membrane immunofluorescence technique, reported that 20 to 35% of PBL from cattle from a leukemia-free herd possessed S-Ig. Eight of twelve clinically normal animals without PL and all nine animals tested with PL from the multiple-case lymphosarcoma BF herd had elevated percentages (43 to 65%) of cells with S-Ig.

Garcia de Lima and Mitscherlich (4) found between 12.6 and 18.6% of lymphocytes in peripheral blood of normal animals to form rosettes in the erythrocyte-antibody-complement (EAC) rosette test. They found that, in lymphocytotic animals, the absolute number of lymphocytes which do not form rosettes increased one-and-a-half times more than the EAC rosette-forming cells (EAC-RFC).

In addition to the reports indicating an increased level of S-Ig cells in PL cattle, Muscoplat et al. (10) reported that unstimulated lymphocytes from cattle with lymphocytosis incorporated approximately five times as much [^{3}H]-thymidine after 3 days in culture as did control cultures from normal animals. Similar findings were also reported by Weiland and Straub (14).

The purpose of this study was to characterize the lymphocyte populations present in cattle infected with BLV and to try to assess their contribution to the lymphocytotic state. To do this animals were selected for study which were free of BLV infection, BLV-infected without exhibiting PL, or BLV-infected with PL. PBL were analyzed in terms of surface markers, whether or not they replicated BLV, and

whether they spontaneously incorporated [^{3}H]-thymidine in culture.

MATERIALS AND METHODS

Animals. Since age does not appreciably influence the absolute lymphocyte count after cattle are approximately 54 months old (7), the cattle selected for this study had ages in excess of 54 months (except BF-281, which was 50 months).

BLV-free animals were selected from leukemia-free (6) Guernsey herds BI and BH (3, 7). They had no antibodies to the internal antigens of BLV as detected in the fluorescent antibody test, and their lymphocytes did not induce syncytia (2) when cocultivated with bovine embryo spleen cells. Syncytia-inhibiting antibodies (2) were not present in sera from any of the BLV-free animals tested in this study. Animals with PL have not appeared in either herd in the last 9 years and no cases of histopathologically confirmed lymphosarcoma have ever been reported in these herds.

Animals naturally infected with BLV were selected from Jersey herd BF (3, 7) in which approximately 60% of the animals have PL and over 90% of the animals have BLV antibodies detectable in the fluorescent antibody test (3). All the infected animals used in this study had antibodies to BLV and their PBL-induced syncytia.

Animals with an absolute lymphocyte count, at three consecutive monthly bleedings, greater than 2.5 standard deviations above the normal mean for cattle above 54 months of age in lymphosarcoma-free control herds of the Jersey breed were considered to have PL (7). This represents an absolute lymphocyte count of 7,500/mm^3 or greater.

Preparation of peripheral blood lymphocytes. Lymphocytes were prepared by density gradient centrifugation of heparinized blood (1 unit of sodium heparin per ml [Panheprin, Abbott Laboratories]). Volumes (3 ml) of heparinized blood were diluted with an equal volume of Eagle minimum essential medium (MEM) in 12-ml conical centrifuge tubes and supported on 4 ml of sodium metrizoate/Ficoll (Lymphoprep, Nyegaard & Co., Oslo). The gradients were centrifuged for 30 to 40 min at 400 × *g* at room temperature, and the bands were collected and washed twice in MEM. Lymphocyte suspensions prepared in this way usually contained less than 5% granulocytes and less than 5% dead cells.

Complete blood counts. Blood was collected in tubes containing ethylenediaminetetraacetic acid as an anticoagulant at a concentration of 1 mg/ml of blood. Total leukocyte counts were made on an automatic cell counter (Fisher Autocytometer). Blood films were freshly prepared and stained by the Wright method. Two hundred cells were counted in the differential count using the "battlement" method.

Membrane immunofluorescence. The presence of immunoglobulin on the surface of PBL was detected by the direct membrane immunofluorescence test using a polyvalent (IgM, IgG, IgA) fluoresceinated rabbit anti-bovine gamma globulin (Behring Diagnostics, Somerville, N.J.).

Lymphocytes (10^7) were washed twice in phosphate-buffered saline (0.01 M sodium phosphate-0.015 M NaCl [pH 7.2]) in a 2-ml centrifuge tube, and the pellet was suspended in 0.05 ml of the antiserum. The fluoresceinated antiserum was used at a 1:10 dilution made with a solution of bovine serum albumin-rhodamine (1:50 in phosphate-buffered saline). The cell suspension was resuspended every 10 min during a 30-min incubation at room temperature. After incubation the suspension was washed three times in phosphate-buffered saline and suspended in two or three drops of 50% glycerol. A small circle of Vaseline was made on a clean glass slide, a drop of the cell suspension was placed within it, and a cover slip was gently pressed down on it. Slides were viewed by using a Zeiss fluorescent microscope with dark-field condenser, exciter filter BG-12, barrier filters 50 and 44, and a high-pressure mercury bulb as the light source. Two hundred cells were counted to determine the percentage of cells with membrane fluorescence.

Preparation of EAC rosettes. Sheep blood was drawn into an equal volume of Alsever solution, washed three times in MEM, and resuspended as a 10% suspension in the same medium. A subagglutinating dose of rabbit anti-sheep hemolysin (GIBCO, Grand Island, N.Y.) and fresh horse complement at a final dilution of 1:40 were added to the sheep red blood cell suspension and incubated at 37°C for 1 h. After being washed twice in MEM, the sheep red blood cells were adjusted to a 10% suspension and refrigerated until use.

For the EAC rosette-forming test, 1 drop of the sensitized sheep red blood cell suspension was added to 3 drops of a 5 × 10^6/ml suspension of lymphocytes, incubated at 37°C for 15 min, centrifuged for 5 min at 60 × *g*, and gently suspended in approximately 1.5 ml of 0.2% trypan blue. The percentage of lymphocytes with three or more sheep red blood cells attached was determined from a count of 200 lymphocytes in a hemocytometer. In preliminary experiments 1% or less of bovine lymphocytes was found to bind unsensitized sheep red blood cells.

Preparation of an EAC-RFC-depleted population. Buffy coats were prepared from heparinized blood by centrifuging 50-ml volumes at 900 × *g* for 35 min at 5°C. The plasma was aspirated and discarded and the buffy coat was collected with a Pasteur pipette. The red blood cells contaminating the buffy coat preparations were lysed with distilled water, and the tonicity was restored after 15 s with a 0.15 M NaCl solution. The cells were then centrifuged at 200 × *g* for 10 min, suspended in MEM, and washed twice more at 200 × *g*.

EAC rosettes were formed by incubating 3 ml of the buffy coat cells (50 × 10^6 to 80 × 10^6/ml) with 2 ml of a 10% suspension of sensitized sheep red blood cells for 30 min at 37°C in a plastic centrifuge tube (16 by 125 mm). The suspension was then centrifuged at 60 × *g* for 10 min, gently resuspended, and supported on 3 ml of Lymphoprep. The tubes were centrifuged at 400 × *g* for 30 min at room temperature, and the interface band was collected, washed twice, and suspended in MEM. This cell suspension is referred to as EAC-RFC depleted. The PBL con-

trols were prepared by identical treatment of buffy coat cells using unsensitized, instead of antibody- and complement-treated, sheep red blood cells.

Syncytia assay for the detection of BLV-infected lymphocytes. Diglio and Ferrer (2) recently showed that BLV-infected bovine lymphoid cells, as well as BLV itself, induce syncytia in several monolayer cell cultures. Syncytia induction in bovine embryo spleen monolayers was used to test PBL and EAC-RFC-depleted PBL for the presence of BLV-infected cells.

Bovine embryo spleen cells (4×10^5 to 5×10^5) in 4 ml of MEM supplemented with 10% fetal calf serum were incubated overnight in Integrid tissue culture dishes (60 by 15 mm; Falcon Plastics, Oxnard, Calif.). The medium was then replaced with 4 ml of medium containing 25 μg of diethylaminoethyl (DEAE)-dextran. After 30 min of incubation at 37°C the medium containing DEAE-dextran was removed and 5×10^6 lymphocytes in 4 ml of fresh medium was added. After 48 h of incubation in a humidified atmosphere containing 5% CO_2, the dishes were rinsed twice and replenished with new medium. After a further 3-day incubation period the monolayers were rinsed twice with phosphate-buffered saline, fixed in absolute methanol, and stained with Giemsa. The number of syncytia present in 100 squares of the grid formed in the plate were counted. A syncytium was defined as a cell containing five or more nuclei.

Presence of antibodies against internal antigens of BLV. Demonstration of antibodies to internal antigens of BLV was accomplished by an indirect fluorescent antibody technique, as described by Ferrer et al. (3). Briefly, the test serum was incubated on an acetone-fixed smear containing known antigen-positive cells. After further incubation with fluorescein isothiocyanate-conjugated goat anti-bovine 7*S* globulin and bovine serum albumin-rhodamine counterstain, the slide was examined for the presence of fluorescent cells. Control smears were incubated with known positive or negative sera or with phosphate-buffered saline before addition of the secondary reagent.

Lymphocyte cultures. PBL were cultured in MEM with a 10% supplement of pooled serum from BLV-free cattle (inactivated at 56°C for 30 min).

Lymphocytes (2×10^5) were cultured in triplicate wells in a volume of 0.2 ml in disposable multi-well tissue culture plates (LINBRO IS-MRC-96-TC). Sixteen hours before termination of the cultures, 0.25 μCi of [^{3}H]thymidine ([methyl-^{3}H]thymidine, specific activity 15.1 Ci/mmol, Amersham/Searle, Arlington Heights, Ill.) in 0.05 ml was added to each well. The cultures were harvested with a multiple automated sample harvester (Cell Harvester model M24, Biomedical Research Institute, Rockville, Md.); the glass fiber filters (grade 934AH, Reeve Angel, Clifton, N.J.) were washed thoroughly with 0.015 M NaCl solution, dried, and placed in liquid scintillant and counted in a Packard Tri-Carb liquid scintillation counter. Incorporation of [^{3}H]thymidine is reported as the mean counts per minute of the triplicate cultures. Standard error of the mean for each set of triplicate cultures was less than 15%.

RESULTS

Relationship between BLV infection, persistent lymphocytosis and the percentage of S-Ig-bearing and EAC-RFC in peripheral blood. Complete blood counts and determination of the percentage of PBL forming EAC rosettes and carrying surface immunoglobulin detectable by membrane fluorescence were carried out on 14 BLV-free animals from leukosis-free herds, 8 BLV-infected animals without PL, and 6 BLV-infected animals with PL. Table 1 shows these results and also the range of lymphocyte counts and percentages of EAC-RFC and S-Ig for each of the groups. An excellent correlation is seen between the percentages of EAC and S-Ig-carrying cells within each group. These data do not provide evidence for a substantial lymphocyte population lacking one of these markers, but not the other, in BLV-infected cattle.

Among the BLV-infected animals without PL, four (BF-099, -147, -231, -248) had total lymphocyte counts less than the mean (4,495/mm^3) for the BLV-free groups. The percentage of lymphocytes with surface markers in these animals is close to that of the BLV-free group. The four remaining animals in this group have lymphocyte counts higher than the mean for the BLV-free animals and tend to have substantially higher percentages of EAC-RFC and S-Ig-bearing lymphocytes. In animals with PL, the average of lymphocytes bearing surface immunoglobulin (66.2%) agrees closely with the results obtained by other investigators (12, 15), but the percentage of EAC-RFC (70.8%) is higher than that observed by Garcia de Lima and Mitscherlich (4).

Figure 1 shows the percentage of EAC-RFC and S-Ig-bearing cells, obtained from Table 1, plotted against a logarithmic representation of the absolute lymphocytes per cubic millimeter. Regression analysis by the least-squares method shows that for BLV-free cattle the percentages of EAC-RFC and S-Ig-bearing lymphocytes are independent of the total lymphocyte count ($P = 0.411$ and 0.430). In BLV-infected animals there is a very strong correlation between the percentage of cells bearing these surface markers and the absolute lymphocyte count ($P < 0.0001$). Cells with surface markers characteristic of B lymphocytes are responsible for most of the increase in circulating lymphocytes accompanying BLV infection.

Effect of removal of EAC-RFC on syncytia induction by peripheral blood lymphocytes. PBL were depleted of EAC-RFC by density gradient centrifugation of EAC rosetted buffy coat cells on Lymphoprep. The PBL and EAC-RFC-

TABLE 1. *Individual lymphocyte counts and percentages of EAC-RFC and S-Ig-bearing lymphocytes in BLV-free and BLV-infected cattle*

Animal	Lymphocytes/mm³		Lymphocyte count range	EAC-RFC (%)	% EAC-RFC range	S-Ig (%)	% S-Ig range
BLV-free							
BH-031	5,506	(1.5)[a]		18		19	
BH-073	3,185	(1.0)		17		25	
BH-095	3,021	(1.0)		13		15	
BH-097	6,336	(1.6)		10		16	
BH-110	4,956	(1.4)		20		30	
BH-116	3,111	(1.0)		16		20	
BH-119	4,509	(1.3)		11		23	
BH-120	4,048	(1.2)	2,562–6,336	16	10–27	19	15–30
BH-121	3,983	(1.2)		16		27	
BH-126	4,583	(1.3)		23		24	
BH-128	6,030	(1.6)		21		26	
BI-196	2,562	(0.8)		21		15	
BI-434	5,328	(1.5)		18		24	
BI-469	5,772	(1.5)		24		16	
				27			
BLV-infected without PL							
BF-099	3,456	(1.1)		17		22	
BF-147	3,696	(1.1)		11		12	
BF-231	3,441	(1.1)		21		19	
BF-248	3,782	(1.2)		23		30	
BF-243	7,448	(1.7)	3,441–7,448	36	11–56	45	12–50
BF-258	6,885	(1.7)		56		50	
BF-277	7,344	(1.7)		38		46	
BF-281	5,056	(1.4)		33		30	
BLV-infected with PL							
BF-157	19,360	(2.6)		78		70	
BF-187	20,886	(2.6)		62		54	
BF-198	11,137	(2.1)		55		55	
BF-223	15,717	(2.4)	11,137–21,656	69	55–81	72	54–76
BF-227	21,656	(2.7)		80		70	
BF-237	21,280	(2.7)		81		76	

[a] Logarithmic representation of the lymphocyte count = 2 × log to the base 10 of the lymphocyte count per cubic millimeter minus six. This representation is used to facilitate manipulation of the lymphocyte counts in the least-square regression analysis (7).

depleted cell suspensions were examined for the presence of EAC-RFC and S-Ig-bearing cells, and portions of 5×10^6 viable cells were tested for their ability to induce syncytia.

Table 2 shows that centrifugation of EAC rosetted buffy coat cells on Lymphoprep efficiently removes EAC-RFC from cell suspensions prepared from BLV-free animals and greatly reduces the percentage of EAC-RFC and S-Ig-bearing cells in cell suspensions prepared from BLV-infected cattle. Table 2 also shows that loss of the EAC rosette-forming population results in a marked reduction of the ability of lymphocyte preparations from BLV-infected animals to induce syncytia. The EAC-RFC-depleted populations consistently induced less than 15% of the number of syncytia that the same number of PBL induced, indicating that most, if not all, lymphocytes replicating infectious BLV are EAC-RFC. In these experiments there was no apparent correlation between the number of syncytia and the presence or absence of lymphocytosis in infected animals.

Effect of removal of EAC-RFC on spontaneous thymidine incorporation by bovine lymphocytes after 3 days in culture. Figure 2 compares the spontaneous incorporation of [^{3}H]thymidine at 3 days by PBL from two BLV-infected animals (with and without PL) with that by PBL from three BLV-free controls. Lymphocytes from infected animals with or without PL spontaneously incorporated higher levels of [^{3}H]thymidine than uninfected ani-

mals. Removal of EAC-RFC from the PBL of the BLV-infected animals resulted in levels of incorporation not much greater than those for EAC-RFC-depleted populations from the BLV-free controls.

Thus, in BLV-infected animals the major population of lymphocytes spontaneously incorporating thymidine after 3 days in culture is part of the EAC rosette-forming population. Interestingly, in the BLV-free animals, EAC-RFC depletion resulted in increased incorporation of [^{3}H]thymidine, suggesting the presence of a non-B cell population capable of spontaneously incorporating [^{3}H]thymidine in the peripheral blood of normal cattle.

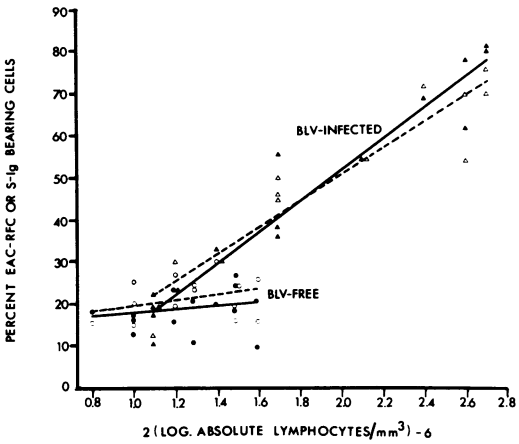

FIG. 1. *Percentage of EAC-RFC and S-Ig-bearing cells in peripheral blood plotted against the logarithmic representation of the lymphocyte count per cubic millimeter. Symbols: ●, BLV-free, EAC-RFC; ○, BLV-free, S-Ig-bearing cells; ▲, BLV-infected, EAC-RFC; △, BLV-infected, S-Ig-bearing cells. Least-squares regression analysis was made and regression lines were drawn. Solid lines indicate EAC-RFC; broken lines indicate S-Ig.*

DISCUSSION

PL in cattle has been associated with BLV infection. In herds studied by Ferrer et al. (3), in which multiple cases of bovine lymphosarcoma had occurred, between 80 and 100% of clinically normal animals affected with PL had

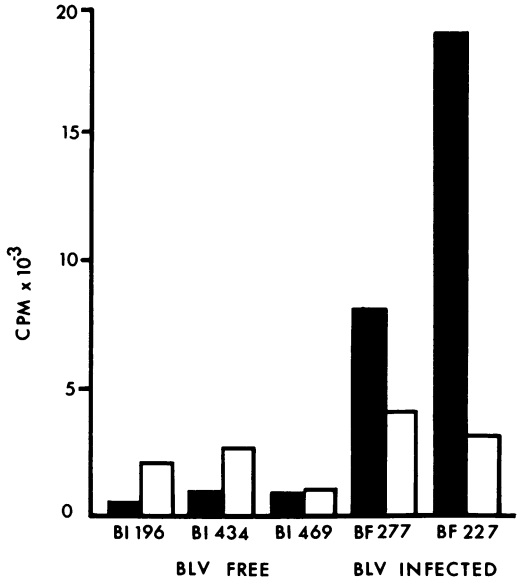

FIG. 2. *Effect of EAC-RFC depletion on the ability of PBL to spontaneously incorporate [^{3}H]thymidine. BI-196, BI-434, and BI-469 are BLV-free; BF-277 and BF-227 are infected with BLV. Symbols: ■, PBL; □, EAC-RFC depleted PBL.*

TABLE 2. *Effect of removal of EAC-RFC on syncytia induction by peripheral blood lymphocytes*

Animal	EAC-RFC (%)		Cells with S-Ig (%)		Syncytia[a]	
	PBL	EAC-RFC-depleted PBL	PBL	EAC-RFC-depleted PBL	PBL	EAC-RFC-depleted PBL
BLV-Free						
BI-196	15.0	1.0	15.0	1.0	0	0
BI-434	15.0	1.0	33.1	15.7	0	0
BI-469	13.5	1.0	25.5	4.0	0	0
BLV-infected without PL						
BF-277	54.6	11.4	62.8	5.5	14	2
BF-099	16.0	ND	25.0	1.0	212	10
BLV-infected with PL						
BF-157	76.9	8.8	65.6	23.5	438	36
BF-187	70.0	16.1	70.0	27.2	15	0
BF-227	86.2	20.8	81.6	28.7	4	0
BF-258	73.0	10.7	65.5	14.4	5	0

[a] Number of syncytia counted in 100 squares of Integrid tissue culture dish (60 by 15 mm) containing BESP monolayer cocultivated with 5×10^6 lymphocytes.

antibodies to BLV internal antigen. In the same herds the incidence of infection with BLV in animals without PL ranged from 25 to 76%. Not only was BLV infection more prevalent in the lymphocytotic cattle but in general the groups with PL had higher BLV antibody titers than those without.

The relationship between PL and histopathologically confirmed bovine lymphosarcoma is not so clear. Bendixen (Ph.D. thesis, University of Copenhagen, Denmark, 1963) observed that 90 to 95% of animals with leukotic tumors had shown PL and proposed that PL is a subclinical form of bovine leukosis. In the herds studied by Abt et al. (1), 66% of the lymphosarcomatous cattle had a history of PL. However, in that study herds were observed in which lymphosarcoma occurred without lymphocytosis, and in a number of herds PL occurred without reported cases of lymphosarcoma (7). Although both lymphosarcoma and PL occur in familial clusters, these do not always coincide (1). Furthermore, a great many animals with lymphocytosis never develop lymphosarcoma, even when kept to advanced ages. Thus, although PL is associated with both BLV infection and the tumor-bearing state, the precise nature of this relationship is unclear. It is apparent that clarification of the role of PL in BLV infection and leukemogenesis requires identification of the cells involved in lymphocytosis and analysis of their functions.

Our studies support the conclusion of other investigators that cells bearing surface markers typical of B lymphocytes predominate in lymphocytosis and indicate that these cells account for most of the increase in lymphocytes circulating in peripheral blood. In direct comparisons of the percentage of EAC-RFC and S-Ig-bearing cells in both normal and BLV-infected animals, we did not find evidence for the presence of a substantial population bearing one marker but not the other.

Our data also indicate that the cells which replicate infectious BLV and the cells which spontaneously incorporate thymidine after 3 days in culture are EAC-RFC, since removal of rosetted lymphocytes on density gradients results in loss of these activities from the remaining population. These experiments do not offer information on whether these two activities are functions of the same or of different cell populations. This question is examined in an accompanying report (5).

A population of leukocytes which also spontaneously incorporates thymidine is detectable in the peripheral blood of BLV-free animals. However, in contrast to the spontaneously incorporating cells of BLV-infected animals, its activity is enhanced rather than reduced by removal of EAC-RFC.

In this study a wide range of lymphocyte counts associated with BLV infection was observed. All animals with BLV antibodies had circulating lymphocytes capable of inducing syncytia whether or not they exhibited PL. The fact that four BLV-infected animals had total lymphocyte counts less than the mean of the BLV-free controls clearly indicates that lymphocytosis is not a reliable marker for BLV infection. Similarly the percentage of EAC-RFC and S-Ig-bearing cells for these animals is within the range for the BLV-free animals. An explanation for the wide range of lymphocyte counts in BLV infecton has not been found. It is unlikely that the low absolute counts seen in some animals are because BLV infection is recent and lymphocytosis has not had time to develop. Serological studies on stored sera from the BLV-infected animals in this study indicate that all had been infected with BLV for at least 36 months. It has been suggested that whether PL occurs or not may depend on the virus dose and/or genetic susceptibility (3). If lymphocytosis is under genetic control, then it would appear that these genetic factors regulate a B cell population and affect either the expansion of a virus-infected population or a lymphocyte population not infected with BLV but expanded in response to BLV infection.

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