Altered Interleukin-2 Production by Lymphocyte Populations from Bovine Leukemia Virus-Infected Cattle (43815)

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Abstract. **The effects of bovine leukosis virus (BLV) on the phenotypic and functional characteristics of peripheral blood mononuclear cells were investigated. Whole blood differentials showed that persistently lymphocytotic (BLV+PL) dairy cattle had more lymphocytes and fewer neutrophils than the aleukemic seropositive (BLV+AL) or seronegative (BLV−) animals. Flow cytometric analyses of peripheral blood mononuclear cells indicated that the BLV+PL animals had more B lymphocytes, with a concomitant decrease in CD2 positive cells when compared with the BLV− group. Mononuclear cells from the BLV+AL animals also had fewer CD2 positive cells, but no differences in B lymphocytes were observed when compared with BLV− cattle. Peripheral blood mononuclear cells were used in blastogenesis assays to assess the functional ability of lymphocytes. Lymphocytes from BLV+PL animals had lower proliferative responses to concanavalin A and pokeweed mitogen when compared with cells from the BLV− or BLV+AL groups. The level of spontaneous blastogenesis in the absence of mitogenic stimulation was high for lymphocytes obtained from BLV+AL cattle. Cultures of lymphocytes obtained from BLV+PL animals produced greater amounts of interleukin-2 (IL-2) than BLV+AL and BLV− groups, although no differences were observed in the expression of IL-2 receptors. The development of uncontrolled lymphocytosis in BLV-infected cattle may result from an altered responsiveness to IL-2-regulated B-lymphocyte proliferation.** [P.S.E.B.M. 1994, Vol 207]

Enzootic bovine leukosis is a contagious disease of cattle induced by an exogenous retrovirus, bovine leukemia virus (BLV). The disease complex is characterized by a persistent lymphocytosis which can culminate in B cell lymphoma (1). Although BLV is associated mainly with infections of B lymphocytes, researchers also have reported the presence of BLV provirus in the DNA of immunoaffinity purified T lymphocytes from BLV-infected cattle (2). Bovine leukemia virus infects between 10%–30% of dairy cows in the United States (3). Most infected cows never show outward signs of disease, and these animals are referred to as asymptomatic or aleukemic. Approximately 30%–70% of BLV carriers will develop a persistent lymphocytosis while fewer than 10% of cows ever develop malignant lymphosarcoma (1, 3, 4).

The effect of BLV infection on bovine health and productivity is somewhat unclear. Some studies have found no influence on milk production, incidence of mastitis, or reproductive performance (5). In contrast, others have found negative effects on BLV infection on cow longevity, reproductive performance, and milk production (6). It is possible that BLV infection may alter normal immune cell functions and decrease host resistance to other diseases. Unfortunately, little is known about the effects of BLV infection status on the immunocompetence of dairy cattle. In earlier studies, mitogen-induced lymphocyte blastogenesis assays were used as an indicator of the functional ability of lymphocytes from BLV-infected and seronegative cattle (7, 8). Infection of T lymphocytes by BLV did not appear to alter the overall response of the lymphocyte

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populations to mitogenic stimuli. However, high levels of spontaneous blastogenesis in the absence of mitogenic stimulation were observed for lymphocyte preparations from both aleukemic and persistently lymphocytotic animals (7, 9). It was suggested that sera from BLV-infected cattle contained a heat-stable blastogenesis-augmenting factor that was capable of enhancing lymphocyte mitogenic responses (7). However, no further attempts were made to identify this putative serum factor.

The purpose of the present investigations was to assess the effects of BLV infection status on the phenotype and function of bovine peripheral blood mononuclear cells. This paper describes shifts in lymphocyte subpopulations in BLV-infected cattle and relates these changes to essential immune cell functions including lymphocyte blastogenesis and cytokine production. The possibility that altered interleukin-2 (IL-2) production and IL-2 receptor (IL-2R) expression may contribute to the development of persistent lymphocytosis is discussed.

Materials and Methods

Animals. A total of 16 mature (at least 3 years old), lactating dairy cows from the Center for Mastitis Research Herd were used in this study. The BLV infection status was monitored by detection of serum antibodies to BLV structural antigens using a double immunodiffusion assay (10). Of the 16 cows tested, seven were BLV seronegative (BLV−), seven were BLV seropositive with no clinical signs of disease (aleukemic, BLV + AL), and 2 were BLV seropositive with a persistent lymphocytosis (BLV + PL). The criteria used to establish persistent lymphocytosis was the detection of $>7.5 \times 10^3$ lymphocytes/mm^3 of blood for more than 3 months.

Isolation of Cells. Sixty milliliters of blood were collected by jugular venipuncture and mixed with acid citrate dextrose at a final concentration of 10%. Blood cells were separated from plasma by centrifugation, suspended in Hanks' Balanced Salt Solution (HBSS), and layered onto Ficoll-Paque (Pharmacia, Piscataway, NJ) with a specific density of 1.077 g/ml. After centrifugation at 400*g* for 30 min, peripheral blood mononuclear cells (PBMC) from the plasma-Ficoll interface were recovered, washed in HBSS, and viable cell numbers were determined by trypan blue dye exclusion. Total white blood leukocyte cell (WBC) counts were made on an automatic cell counter (Coulter Electronic Ltd., Hialeah, FL) and differential counts were conducted on duplicate blood smears with Wright's-Giemsa staining. Total lymphocyte counts were estimated by multiplying the percentage of lymphocytes in 100 leukocytes counted from the blood smear.

Flow Cytometry. Isolated PBMC were suspended to 4.0×10^7 cells/ml in phosphate buffered saline with 0.2% gelatin (PBSG). Cells (50 μl) were incubated in a 96-well microtiter plate for 30 min at 4°C with 50 μl of monoclonal antibodies specific for bovine leukocyte antigens (VMRD, Pullman, WA). Cells from each cow were incubated with each of the following lineage-specific monoclonal antibodies: B26A (pan T lymphocyte, CD2 antigen), CACT83B (T helper lymphocyte, CD4 antigen), CACT80C (T cytotoxic/suppressor lymphocyte, CD8 antigen), DH59B (granulocyte/monocyte antigen), BAQ44A (B lymphocytes, B-B2), B7A1 (γδ T lymphocytes, WC1), TH14B (MHC Class II molecules), and CACT116A (IL-2R). Cell surface markers were visualized with goat anti-mouse F(ab)2 IgG-fluorescein isothiocyanate (Becton Dickinson Immunocytometry Systems, San Jose, CA) using a staining procedure described previously (7). Appropriate control groups were included to detect nonspecific labeling due to Fc receptor binding. Following labeling, all samples were fixed in a 2% solution of formaldehyde in PBSG and stored in the dark at 4°C. All analyses were performed with an EPICS CS (Coulter Electronics Ltd., Hialeah, FL) and immunofluorescence histograms were expressed as percentage of positive-staining cells. Absolute numbers of T and B lymphocytes were determined by multiplying the percentages of these cells by the total lymphocyte counts.

Lymphocyte Proliferation Assays. The ability of PBMC to proliferate in response to various mitogenic stimuli was assessed. Peripheral blood lymphocytes were cultured in RPMI 1640 supplemented with 25 m*M* HEPES (GIBCO, Grand Island, NY), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 2 m*M* glutamine (Sigma Chemical Co., St. Louis, MO), and fetal bovine serum (FBS) (Hyclone Labs, Logan, UT). All cultures were incubated at 37°C with 5% CO_2. In triplicate wells, 100 μl of PBMC (2×10^6 cells/ml) were combined with optimal concentrations of either concanavalin A (ConA) (10 μg/ml) or pokeweed mitogen (PWM) (0.1 μg/ml) in 100-μl volumes. Background proliferation was determined with PBMC cultured in RPMI-1640 supplemented with 10% FBS. After a 48-hr incubation, 0.4 μCi of [methyl-^{3}H]thymidine (^{3}HTdr) in 20 μl RPMI 1640 was added to each well and incubated for an additional 18 hr. Cells were harvested with an automated harvester (Skatron, Sterling, VA) onto glass filter mats and thymidine incorporations were determined with a Beckman LS 6000IC liquid scintillation counter (Beckman, Palo Alto, CA). The incorporation of ^{3}HTdr was expressed as mean counts per minute (cpm) per culture. Results are expressed as increased cpm compared with background counts.

Interleukin-2 Production and Assay. Isolated PBMC were suspended to 1×10^7 cells/ml of RPMI 1640 supplemented with 10% FBS. In duplicate wells of a 48-well plate, 0.5 ml of PBMC were combined with 0.5 ml of RPMI supplemented with 10 μg/ml of Con A. Duplicate control cultures were supplemented with RPMI-1640 without mitogen. Cultures were incubated for 24, 48, and 72 hr before supernatant were collected, treated with α-D-mannopyranoside, and stored frozen (−20°C) until assay. The cells were collected and stained for the presence of IL-2R expression using flow cytometry.

The IL-2 activity in culture supernatant was measured in a proliferation assay using ^{3}HTdr incorporation by IL-2 dependent bovine T lymphocytes. Dependent cells were prepared by stimulating PBMC cultures with ConA and phorbol 12 myristate 13 acetate. Flow cytometric analysis indicated that the majority of the cells were of the CD4+ phenotype with approximately 80% of the population expressing IL-2R. The IL-2-dependent cells were suspended in RPMI 1640 supplemented with 10% FBS, 0.1 m*M* 2-mercaptoethanol, and 10 mg/ml of methyl α-D-mannopyranoside to a final concentration of 1×10^5 cells/ml. In triplicate wells, 100 μl of the IL-2-dependent cells were cultured with serial dilutions (½ to 1/16) of the IL-2 containing ConA culture supernatant (100 μl). Plates were incubated for 24 hr, 0.4 μCi of ^{3}HTdr in 20 μl of RPMI were added to each well, and the cultures were incubated for an additional 18 hr. Incorporation of thymidine into cellular DNA was assessed following the harvest of cells onto glass fiber filter mats. The incorporation of ^{3}HTdr was expressed as mean cpm for each culture. The IL-2 activity in culture supernatant was estimated by extrapolation from a standard curve generated with known concentrations of recombinant bovine IL-2 (CIBA-GEIGY Ltd., St. Aubin, Switzerland).

Statistical Analysis. Statistical analysis was carried out using a general linear model (GLM) procedure in SAS. Data for IL-2 production was log transformed to comply with homogeneity of variance. For IL-2 activity and IL-2R expression, the experimental design was a split plot with BLV status as the main effect and time as the subplot. The effect of BLV was tested using the cow within BLV status as the error term. All data are reported as least square means ± SEM.

Results

Repeated routine blood tests indicated that both the BLV− and BLV+AL animals had total WBC within normal ranges, while BLV+PL animals were typically twice as high (Table I). Whole blood differentials showed that BLV+PL animals had a significantly higher ($P \leq 0.05$) proportion of lymphocytes and fewer neutrophils than BLV− and BLV+AL animals which were within normal ranges. Absolute lymphocyte counts from BLV+PL cattle were higher (14.15×10^3 cells/mm^3) than those of the BLV+AL (5.74×10^3 cells/mm^3) of BLV− (4.98×10^3/mm^3) groups.

Shifts in peripheral blood lymphocyte subpopulations were examined in BLV-infected and BLV− cattle using flow cytometric analyses (Fig. 1). The significant increase ($P \leq 0.05$) in lymphoid populations from BLV+PL animals was due to a higher absolute number of B lymphocytes (9.82×10^3 cells/mm^3) when compared with the BLV− (1.24×10^3 cells/mm^3) and BLV+AL (2.01×10^3 cells/mm^3) groups. The absolute numbers of T lymphocytes from both the BLV+AL (1.64×10^3 cells/mm^3) and the BLV+PL (1.10×10^3 cells/mm^3) groups were significantly lower ($P \leq 0.05$) than the BLV− (2.25×10^3 cells/mm^3) animals with significantly lower ($P \leq 0.01$) proportion of CD4+, CD8+, and WC1+ cells. The CD4:CD8 ratio of the BLV+PL (1.90) group was higher than either the BLV+AL (1.52) or BLV− (1.51) groups, although this difference was not significant ($P \geq 0.05$). The BLV+PL cattle also had a higher proportion of cells expressing MHC Class II molecules with a concomitant decrease in the proportion of cells carrying monocyte/granulocyte markers when compared with either BLV− or BLV+AL animals.

The effects of BLV infection status on the spontaneous (PBMC cultured with no mitogen) and mitogen-induced proliferate responses of lymphocyte are summarized in Figure 2. The BLV+PL animals had significantly lower ($P \leq 0.05$) *in vitro* proliferative responses to ConA and PWM than the other two groups. There were no significant ($P \leq 0.05$) differences in the

Table I. Total and Differential WBC Counts from BLV-Infected and BLV− Cattle

Infection status	WBC count (cells/mm^3)	Cell type (%)		
		Lymphocytes	Monocytes	Granulocytes
BLV− (n = 7)	9.52×10^3	52.43 ± 3.27^a	3.71 ± 0.05^a	43.86 ± 2.80^a
BLV + AL (n = 7)	9.71×10^3	59.14 ± 3.27^a	4.42 ± 0.50^a	36.43 ± 2.80^{a,b}
BLV + PL (n = 2)	18.5×10^3	76.50 ± 6.17^b	3.00 ± 0.94^a	20.50 ± 5.23^b

Note. Data are expressed as mean percent ± SE. Percentages within each cell type with a different superscript are significantly different ($P \leq 0.05$).

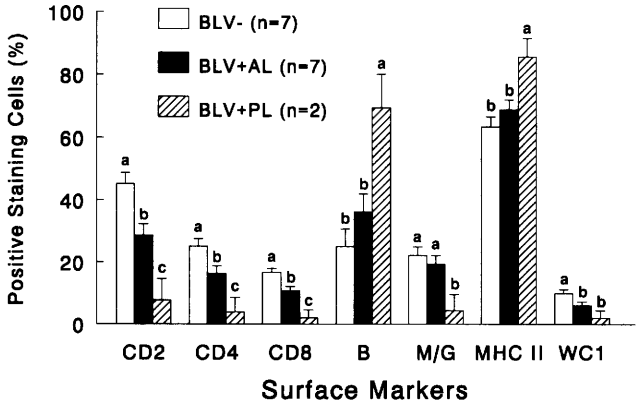

Figure 1. Flow cytometric profile of isolated PBMC from BLV−, BLV+AL, and BLV+PL cattle. Data are expressed as mean percentage of positive-staining cells for each specific surface marker detected by monoclonal antibodies. Means within each surface marker with different superscripts are significantly different ($P \leq 0.05$).

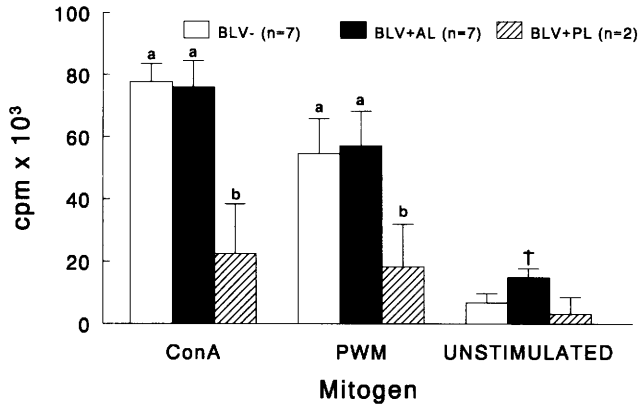

Figure 2. Proliferative responses of PBMC obtained from BLV−, BLV+AL, and BLV+PL cattle when stimulated with ConA (10 mg/ml), PWM (1 mg/ml), or unstimulated (no mitogens) as measured by ^{3}HTdr incorporation. Data are expressed as mean corrected cpm for each treatment group except the unstimulated group. Means within each mitogen with different superscripts are significantly different ($P \leq 0.05$). †Means for the BLV+AL unstimulated cells are significantly higher ($P \leq 0.06$).

mitogenic responses between BLV+AL and BLV− animals. However, the spontaneous proliferation of PBMC from BLV+AL cattle was significantly higher when compared with that for the BLV− group.

Changes in IL-2 production and IL-2R expression were measured after 24, 48, and 72 hr *in vitro* stimulation with ConA. The BLV+PL animals produced greater amounts of IL-2 than the other two groups at all time points (Fig. 3). Receptor expression for all cows increased after stimulation with ConA, but did not change significantly ($P \geq 0.05$) after 24 hr. There were no differences in IL-2R expression among the three groups at any time point (data not shown).

Discussion

Results of this study show that there are differences in phenotypic and *in vitro* functional capabilities

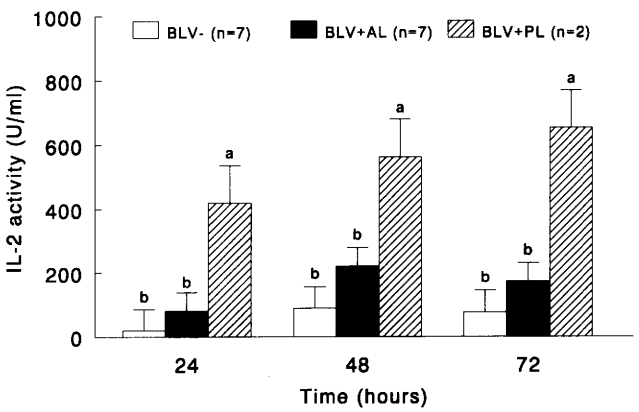

Figure 3. The level of IL-2 activity in ConA-stimulated cell culture supernatants of PBMC obtained from BLV−, BLV+AL, and BLV+PL cattle. Cells were stimulated for 24, 48, and 72 hr before harvest of the supernatants for assay. Means within each time period with different superscripts are significantly different ($P \leq 0.05$).

among BLV−, BLV+AL, and BLV+PL animals. The trends seen in total and differential WBC counts agree with those seen in earlier studies. Cattle are considered lymphocytotic if blood lymphocyte counts exceed 7.5×10^3 cells/mm^3 (1, 11). The number of lymphocytes in the blood of BLV− and BLV+AL animals did not exceed this limit, while counts from the BLV+PL cattle were well over 7.5×10^3 cells/mm^3.

Flow cytometric analyses were used to further characterize absolute numbers of B and T lymphocytes in BLV-infected cattle. Data presented in this paper agree with the results reported previously showing a significant increase in B lymphocytes with a corresponding decrease in total T lymphocytes of both CD4+ and CD8+ subsets in the peripheral blood of BLV+PL cattle (12, 13). This study further showed a reduction in the proportion of γδ T lymphocytes in both the BLV+AL and BLV+PL groups when compared with the normal animals. However, there are some conflicting reports regarding the numbers of circulating B and T lymphocytes in BLV+AL animals. Although total and differential WBC counts were within normal range for the BLV+AL animals in the present study, a significant decrease in T lymphocytes with reduced numbers of CD4+, CD8+, and γδ T lymphocytes subsets was observed. Numbers of B lymphocytes tended to be higher than the levels observed in the BLV− group, but the difference was not significant. These findings are in contrast to the results of previous studies in which either no leukocyte perturbations (7) or low lymphocyte counts associated with reduced numbers of both B and T lymphocytes (12, 13) were noted in BLV+AL animals.

A likely explanation for this conflict between different studies may be related to the length of time cattle were infected with BLV. Experimental BLV infection of sheep revealed a progressive shift in leuko-

cyte populations over a 10-month period where both T and B lymphocyte populations increased initially following infection, but by the end of the trial, the numbers of T lymphocyte decreased significantly (14). The experimental animals used in the present study seroconverted BLV positive at least 12 months prior to sample collection. Therefore, the profile of circulating lymphocytes described in this study may reflect the distribution of B lymphocytes and T lymphocyte subsets in BLV + AL cattle during later stages of infection.

Lymphocytes respond to mitogenic stimulation by producing cytokines, expressing cytokine receptors, and ultimately proliferating. Although lymphocyte blastogenesis is not a specific effector function of lymphocytes, this assay is used routinely to assess the overall immunocompetence of an animal. Proliferation in response to ConA is a property of thymus-derived lymphocytes, while responsiveness to PWM is a property of B lymphocytes in the presence of T cell-derived cytokines (9). Results from the present study corroborate earlier findings that demonstrated a reduction in mitogen-induced proliferation of PBMC from BLV + PL animals (15). A significant reduction in proliferation was evident in both ConA − and PWM-stimulated cultures from BLV + PL animals when compared with BLV − and BLV + AL groups. In accordance with previous studies (7), no significant differences were noted in the ability of lymphocytes from BLV − and BLV + AL animals to respond to mitogen stimulation with optimal concentrations of either ConA or PWM. This was surprising, since there were significantly fewer T lymphocytes (CD4 + and CD8 +) in the mononuclear cell preparations of BLV + AL used for the proliferation assays. Since the ability of B lymphocytes to respond to PWM is dependent on T helper lymphocytes, one might also expect to see a decreased responsiveness to this mitogen. These results suggest that T lymphocytes from BLV + AL animals may be more responsive to mitogenic stimulation than the cells from BLV − animals. In support of this hypothesis, spontaneous proliferation in the absence of mitogen stimulation by cells from BLV + AL animals were significantly higher than cells from the BLV − group. The observation that lymphocytes from BLV + AL animals have high levels of spontaneous blastogenesis was reported previously, and it was suggested that these cells may be responding to certain serum factors present in BLV-infected animals (7). In fact, BLV-positive sera was shown to enhance the mitogenic responses of lymphocytes from BLV − animals to ConA, PWM, and phytohemagglutinin (7).

It is not known what factor(s) are responsible for the uncontrolled lymphocytosis associated with BLV. Recent studies demonstrated the presence of BLV genomic DNA in T helper and T cytotoxic/suppressor lymphocyte cultures from BLV + AL and BLV + PL cattle (2, 16). The BLV genome encodes a viral transactivator transcriptional protein, *tax* (17). This protein may be expressed after viral integration into T helper lymphocytes and modify the expression of genes encoding B lymphocyte growth factors, such as cytokines. Interleukin-2 is one of the many T-lymphocyte-derived cytokines of major importance in the regulation of a variety of immune cell activities including initiating and sustaining proliferation. The production of IL-2 is an autocrine feedback loop in which binding of IL-2 leads to increased IL-2R expression by T lymphocytes with a subsequent increase in IL-2 release. The autocrine actions of IL-2 suggest that this cytokine may be involved in oncogenesis and possibly leukaemogenesis. In this study, the levels of IL-2 activity were greatly enhanced in BLV + PL animals and continued to increase as length of *in vitro* culture increased. However, the expression of IL-2R was similar for all three groups. This suggests that maximum expression of IL-2R is achieved within 24 hr of addition of ConA regardless of BLV infection status. The significantly higher levels of IL-2 activity in BLV + PL lymphocyte cultures without a concurrent elevation in IL-2R expression could be due to a deficiency in the IL-2R internalization process or the loss of IL-2 dependence. Failure of PBMC to respond to IL-2 may explain the decreased proliferative responses of BLV + PL lymphocyte populations to ConA and PWM as was seen in this study.

In support of this hypothesis, a similar human retrovirus (human T-cell leukemia virus, HTLV-1) was shown to interfere with normal lymphocyte responsiveness to autocrine or paracrine stimulation by IL-2. The two viruses (BLV and HTLV-1) share similar gene structural arrangements with significant homology in several regions including the gene complex which encodes the *tax* protein. Researchers have shown that the *tax* protein encoded by HTLV-1 can increase transcription of the cellular genes encoding IL-2 and IL-2R (18). It was suggested that HTLV-1 eventually causes a breakdown of the autostimulatory loop with the loss of IL-2 dependence and resulting in the uncontrolled proliferation of lymphoid cells (19). Although the factor(s) that mediate the development of persistent lymphocytosis and uncontrolled cell proliferation following BLV seroconversion are unknown, a mechanism similar to the HTLV-1 may be responsible. Future studies will examine the effects of BLV on the potential breakdown of growth control mechanisms associated with IL-2 and the IL-2R at the molecular level.

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