Spontaneously proliferating lymphocytes from bovine leukaemia virus-infected, lymphocytotic cattle are not the virus-expressing lymphocytes, as these cells are delayed in G₀/G₁ of the cell cycle and are spared from apoptosis

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Bovine leukaemia virus (BLV) is in the family of oncogenic retroviruses which includes human T cell leukaemia virus (HTLV). BLV infects B lymphocytes and induces a non-neoplastic persistent lymphocytosis (PL) of B lymphocytes in cattle. A characteristic of BLV- and HTLV-induced disease is spontaneous lymphocyte proliferation of cultured peripheral blood mononuclear cells (PBMC). To investigate the role of virus expression on lymphocyte survival and proliferation, we evaluated cell cycle position, apoptosis and virus expression on a single-cell basis of cultured PBMC from BLV-infected PL cattle, BLV-infected non-PL cattle and uninfected cattle. Results demonstrated that the majority of bovine B lymphocytes spontaneously entered G₂/M of the cell cycle and died by apoptosis by 24 h post-culture, regardless of BLV infection or PL status. The spontaneous proliferation that characterizes PL cattle was primarily due to a small population of surviving B lymphocytes, but T lymphocytes also contributed. Viral protein expression was detectable in only 5–15% of cultured PBMC from PL cattle and the majority of these lymphocytes were delayed in cell cycle and spared from apoptosis. Unexpectedly, we determined that only 3% of the spontaneously proliferating lymphocytes expressed viral proteins. Previous reports show that spontaneous proliferation decreases when virus expression is suppressed. Together with our results, this suggests that virus expression by one population of B lymphocytes promotes proliferation of another population of B lymphocytes that does not express virus. This may be due to an effect of virus on CD4 T lymphocytes, as depletion of CD4 T lymphocytes significantly decreased spontaneous proliferation.

Introduction

Bovine leukaemia virus (BLV) belongs to a family of oncogenic retroviruses which includes human T cell leukaemia virus (HTLV) I and II and simian T cell leukaemia virus I and II. These retroviruses share a common genomic organization (Sagata et al., 1985) and are associated with non-neoplastic lymphocyte dysregulation, lymphoid neoplasia and/or progressive myelopathies (Uchiyama, 1997). BLV is unique in the HTLV family of retroviruses because it infects and dysregulates B lymphocytes instead of T lymphocytes. Infection with BLV persists for life and results in a persistent lymphocytosis (PL) of predominantly CD5-expressing B lymphocytes in approximately 30% of infected cattle (Depelchin et al., 1989; Burny et al., 1988). PL is a polyclonal, non-neoplastic B lymphocytosis that often precedes the development of B cell lymphoma or leukaemia observed in 1–10% of infected cattle (Burny et al., 1985, 1988).

One hallmark of HTLV and BLV infection is the incorporation of [³H]thymidine by peripheral blood lymphocytes when cultured in the absence of exogenous antigen or mitogen (Itoyama et al., 1988; Prince et al., 1991a, b; Lal et al., 1992; Muscoplat et al., 1974; Richardson et al., 1997; Lal & Rudolph, 1991), referred to as spontaneous lymphocyte proliferation. Because both clinical and lymphocyte phenotypic changes in HTLV and BLV infection are associated with spontaneous lymphocyte proliferation, it is suggested that this

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proliferation is an in vitro correlate of the disease process (Itoyama et al., 1988; Mann et al., 1994). The transition from symptomatic infection to PL in BLV-infected cattle is well defined and there is a clear association between PL and spontaneous lymphocyte proliferation (Thorn et al., 1981; Muscoplat et al., 1974). BLV-induced PL in cattle is also characterized by increased in vivo viral gene expression and a marked increase in provirus-containing B lymphocytes (Mirsky et al., 1993, 1996; Gaynor et al., 1996). Although viral gene expression is restricted in vivo, BLV proteins and virus particles are detected within 3 to 6 h after peripheral blood mononuclear cells (PBMC) from PL cattle are put into culture (Jensen et al., 1990; Baliga & Ferrer, 1977; Dijilali et al., 1987) and thus, prior to peak spontaneous lymphocyte proliferation at 72 h (Jensen et al., 1990; Baliga & Ferrer, 1977). In addition, suppression of in vitro virus expression by either anti-BLV antibody or protein kinase C inhibitors decreases spontaneous proliferation (Trueblood et al., 1998; Thorn et al., 1981; Jensen et al., 1992). Thus, the spontaneous lymphocyte proliferation associated with BLV-induced PL in cattle provides an excellent animal model system to investigate retroviral gene expression and mechanisms of lymphocyte activation and expansion associated with disease progression.

The expansion of B lymphocytes in BLV-induced PL could be due to increased proliferation and/or increased lifespan. To investigate the interaction between BLV expression, cell cycle progression and cell survival, we analysed lymphocyte subset DNA profiles, apoptosis and BLV expression during the time-frame of spontaneous lymphocyte proliferation on a single-cell basis using flow cytometric analysis. Contrary to expectations we found that proliferating lymphocytes did not contain detectable viral proteins and that lymphocytes with detectable expression of viral proteins were more likely to remain in G0/G1 of the cell cycle and were spared from apoptosis.

Methods

**Animals.** Adult Holstein cows used in this study were part of a university dairy and were naturally infected with BLV. A seropositive result by agar gel immunodiffusion to gp51 antigen (Leukassy B; Pittman-Moore) identified BLV-infected cows. BLV-infected, PL cows demonstrated three consecutive white blood cell counts at monthly intervals that were three or more standard deviations above the mean for age-matched, BLV-seronegative cattle (Bendixen, 1965). BLV-infected, non-PL (NPL) cows tested seronegative but maintained normal white blood cell counts. Uninfected (NEG) cows were seronegative for BLV and maintained normal white blood cell counts. Samples were collected from eight PL, three NPL and five NEG cows.

**Monoclonal antibodies.** Mouse monoclonal antibodies (MAbs) were obtained from the Washington State University Monoclonal Antibody Center, Pullman, WA, USA. Listed are the MAbs used in this study, the determinants recognized by these MAbs on bovine mononuclear cells and the MAb isotype. Cell populations were characterized with MAbs directed against the bovine monocyte/granulocyte lineage (DH59B, IgG1), CD14 (CAM36A, IgG2b), bovine B lymphocytes (slgM) (Blg73A, IgG1; Plg45A, IgG2b), the bovine γδ T cell receptor (TCR) (CACT61A, IgM; GB21A, IgG2b), bovine (bo) CD4 lymphocytes (CACT138A, IgG1; GC50A1, IgM), boCD8 lymphocytes (CACT80C, IgG1; 7C2B, IgG2b), boCD3 lymphocytes (MMIA, IgG1), boCD2 (CACT95A, IgG1) and boCD5 lymphocytes (B29A, IgG2b). MAbs used for T lymphocyte depletion by complement-mediated lysis were boCD8 (7C2B, IgG2b), boCD4 (GC50A1, IgM), boCD2 (B26A4, IgM) and bovine γδ TCR (GB21A, IgG1). For CD4 depletion alone MAb boCD4 (GC50A1) was used and for CD8 depletion alone MAb boCD8 (7C2B) was used. Mouse MAbs to BLV capsid protein p24 were obtained as a gift from D. Portetelle, Faculty of Agronomy, Gembloux, Belgium (4’G9, IgG1) and from Veterinary Medical Research Diagnostics (VMRD) (Pullman, WA, USA) (BLV3, IgG1). The gp51 epitope of the BLV envelope glycoprotein was identified with a mouse MAb to the G antigen (BLV1, IgG1; VMRD).

**Lymphocyte isolation, subset depletion, culture and proliferation assays.** Blood was collected by jugular venipuncture in acid citrate dextrose and PBMC were isolated by density gradient centrifugation as previously described (Stone et al., 1995). Viable cells determined by trypan blue dye exclusion were plated at 5 × 10⁶ cells per well in round-bottom 96-well plates (Sarstedt) in complete culture medium (RPMI-1640, antibiotics, 2 mM L-glutamine, 10 mM HEPES buffer supplemented with 20% FBS). Lymphocytes were cultured at 37 °C in 5% CO₂. For lymphoproliferation assays 0.5 µCi [methyl-³H]thymidine (6 mCi/mmol; NEN Life Science Products) was added at the initiation of culture for 24 h assays and for the last 18 h of culture for 72 h assays. Cells were harvested on an automated 96-well plate harvester (Tomtec) and the amount of radioactivity was determined by liquid scintillation spectrometry (Wallac). Data are expressed as the mean and standard deviation (SD) of six replicate samples.

Complement-mediated depletion of T lymphocyte subsets was performed according to our previously published procedure (Stone et al., 1996).

**Flow cytometry.** Single-colour surface staining of cells for flow cytometric analysis was performed using a standard protocol as previously described (Stone et al., 1995). The BLV internal antigen p24 was stained by first fixing cells in 2% formaldehyde in PBS for 20 min on ice, washing once in PBS, followed by permeabilization in 0.2% Tween 20 for 15 min at 37 °C. Following a second wash in PBS, cells were stained with MAbs to BLV p24 (4’G9 or BLV3) by the standard protocol excluding fixation as the final step. Cells were either analysed for single fluorescence by resuspending the cells in PBS with 0.1% sodium azide as the final step or processed further as described below for detection of apoptotic nuclei by the TUNEL method or stained with propidium iodide for cell cycle analysis. Stained cells were enumerated using a FACSsort flow cytometer (Becton Dickinson Immunocytometry Systems) and analysed with CELLQuest or Macintosh Attractors software. Percentages of live, blast-size, and both live and blast-size cell populations were calculated using the forward scatter (FSC) and right angle side scatter (SSC) properties of 2% formaldehyde-fixed cells and either CELLQuest or Macintosh Attractors software. Populations within PBMC were identified as either blast-size or non-blast-size based on the greater linear FSC of blast-size cells. Within the same cultures of PBMC, the live and dead cell populations were separated based on the increased log SSC of dead cells.

**Cell cycle analysis.** Cells were stained with FITC using MAbs to detect slgM (Blg73A) and CD3 (MMIA), permeabilized and stained for cell cycle analysis as previously described (Stone et al., 1995). Surface staining, fixation and permeabilization were carried out in a 96-well plate.
Briefly, following staining for surface markers, cells were fixed with 2% formaldehyde in PBS for 20 min on ice, washed in PBS, permeabilized in 0.2% Triton X-100, and stained with 22 Kunitz U/ml of RNase A. BLV p24 antigen was stained by the procedure described above followed by addition of the propidium iodide/RNase A reagent for cell cycle analysis. 20,000–40,000 events were collected. Cells were gated to exclude debris and include all lymphocyte populations, including those with increased granularity typical of dead cells. Doublets were excluded from the final analysis using linear plots of FL2-A vs FL2-W. Cells were identified as either G4/M cells with a DNA content of 4n or as cells with a DNA content of less than 4n. The 2% formaldehyde fixation procedure used prior to permeabilization prevents leakage of small molecular mass DNA fragments from the nucleus so that total DNA content includes fragmented, apoptotic DNA (Darzynkiewicz et al., 1995).

**Incorporation and detection of BrdU.** 5-Bromo-2′-deoxyuridine (BrdU) (Roche Molecular Biochemicals) was added to the complete medium at a final concentration of 10 μg/ml at the onset of culture. Phycoerythrin (PE) was used to identify MAb binding to IgM (Bgl73A) and CD3 (MMLA). BrdU was detected according to published procedure (Tough & Sprent, 1996). Briefly, cells were surface-stained by the standard protocol omitting the final fixation step, collected from the 96-well plate into 12 × 75 mm tubes in 0.5 ml PBS and fixed in 1:2 ml 95% ice-cold ethanol for 30 min. After washing, cells were resuspended in 1:0 ml formaldehyde fixative (1% formaldehyde, 0.01% Tween 20 in PBS) for 30 min at room temperature. After washing, cells were resuspended in DNase I from bovine pancreas (Sigma) at 50 Kunitz U/ml in 4.2 mM MgCl2/0.15 M NaCl, pH 5.0 and incubated for 10 min at room temperature. Cells were washed in PBS followed by addition of 50 μl anti-BrdU-FITC (Roche Molecular Biochemicals) diluted to 5 μg/ml to resuspended cell pellets. Cells were incubated for 30 min at room temperature, washed, resuspended in PBS containing 0.1% sodium azide and analysed by flow cytometry.

**Detection of apoptotic cells.** Apoptotic cells were detected using the TUNEL technique performed according to the manufacturer’s instructions (ApopTag Kit Fluorescein, Oncor) with the following modifications in fixation and permeabilization. Cells were fixed in 2% formaldehyde for 20 min on ice, followed by permeabilization in 0.2% Tween 20 for 15 min at 37°C. After the permeabilization procedure cells were washed three times in PBS before proceeding with the manufacturer’s instructions for the TUNEL assay. Cell populations identified by PE staining for internal antigens (p24, IgM, CD3) followed the TUNEL-FITC reaction. The propidium iodide/RNase A reagent used to detect the stage of cell cycle concurrent with apoptosis was added following TUNEL-FITC staining.

**Statistical analysis.** Analysis of variance was used to determine significant differences at P ≤ 0.05 in the number of B and T lymphocytes in G4/M between PL, NPL and NEG cow groups at all time-points and between time-points for each of the PL, NPL and NEG groups. For comparison of the two sample means at t = 0 and t = 24 h within a disease group a one-tailed Student t-test was used. For lymphoproliferation assays significant differences between the six replicate samples from non-depleted and CD4-depleted PBMC for each cow were determined by analysis of variance at the P ≤ 0.05. Student’s t-test was used to test for significance in the percentage of p24-expressing lymphocytes at t = 24 h and t = 72 h as well as for the percentage of p24-expressing lymphocytes in G4/M at these time-points.

**Results**

The 72 h spontaneous lymphocyte proliferation that characterizes BLV-infected PL cattle is primarily due to the proliferation of B lymphocytes, but proliferating T lymphocytes also contribute

We and others have shown that unstimulated PBMC from PL cattle spontaneously proliferate as measured by 72 h [3H]thymidine uptake, whereas PBMC from NPL or BLV-infected but NPL cattle do not (Muscoplat et al., 1974; Kenyon & Piper, 1977; Trueblood et al., 1998; Stone et al., 1997). It has been proposed that the spontaneous lymphocyte proliferation that characterizes PL cattle is due to the proliferation of the expanded B lymphocyte population. To more precisely define spontaneous lymphocyte proliferation we first determined the phenotypes of the live, blast-size cells at peak 72 h proliferation by simultaneous analysis of lymphocyte surface antigen expression, cell size and cell viability using flow cytometric analysis (Table 1). In agreement with previous reports PBMC from PL cattle in this study consisted primarily of B lymphocytes (mean of 64%, SD 13%) (Kenyon & Piper, 1977a; Takashima et al., 1977; Kumar et al., 1978; Fossum et al., 1988; Williams et al., 1988; Stone et al., 1997, 1996, 1994). Blast-size cells were identified by an increase in cell size and dead cells were identified by an increase in granularity (see Methods). Because PBMC from NEG and BLV-infected NPL cattle are not characterized by spontaneous proliferation, cells from these animals were not evaluated. Results demonstrated that only a mean of 8% of all PBMC were both live and blast-size at 72 h. Most live, blast-size cells were B lymphocytes (mean of 72%), but T lymphocytes also contributed to this population (mean of 27%). Although the majority of cells in PBMC from PL cattle are B lymphocytes, only 9% of all B lymphocytes in the PBMC cultures were live, blast-size cells at 72 h.

To evaluate by another method the phenotypes of the blast-size lymphocytes, PBMC from one BLV-infected PL cow were cultured with BrdU for 20, 48 or 72 h followed by dual staining for lymphocyte surface antigen expression and BrdU uptake (Fig. 1). Only 4–18% of cells stained positive for BrdU during culture. Consistent with our results on the percentages and phenotypes of the live, blast-sized cells at 72 h, 9% of the B lymphocytes and 4% of the T lymphocytes contained BrdU at this time-point and the majority (86%) of BrdU-staining lymphocytes were B lymphocytes.

It is possible that T lymphocytes from PL cows not only contribute directly to spontaneous lymphocyte proliferation but also promote spontaneous proliferation of B lymphocytes (Stone et al., 1995, 1997, 1994; Trueblood et al., 1998). To explore this possibility 72 h [3H]thymidine uptake was evaluated in PBMC depleted or not depleted of CD4 T lymphocytes (Table 2). When CD4 T lymphocytes were depleted, the remaining cells from PL cattle showed a significant decrease in [3H]thymidine uptake at 72 h whereas
Table 1. Phenotypes of live and live, blast-size cells in 72 h PBMC cultures from eight BLV-infected PL cows

<table>
<thead>
<tr>
<th>Cow no.</th>
<th>PBMC or subset that are live, blast-size cells (%)</th>
<th>Live, blast-size cells (%) that are:</th>
<th>Live cells (%) that are:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBMC</td>
<td>B cells</td>
<td>T cells</td>
</tr>
<tr>
<td>1374</td>
<td>7</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>1379</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>1440</td>
<td>13</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>1570</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>1583</td>
<td>25</td>
<td>27</td>
<td>4</td>
</tr>
<tr>
<td>1602</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1622</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>1713</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Mean</td>
<td>8</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>(SD)</td>
<td>(8)</td>
<td>(9)</td>
<td>(2)</td>
</tr>
</tbody>
</table>

Fig. 1. During spontaneous lymphoproliferation only a small percentage of both B and T lymphocytes incorporate exogenous BrdU. PBMC were surface stained for either sIgM-PE or CD3-PE and positive lymphocytes appear in the upper quadrants (FL2-H). PBMC were internally stained with anti-BrdU-FITC MAb and lymphocytes incorporating BrdU appear in the right-hand quadrants (FL1-H). The numbers in the quadrants of each dot plot indicate the percentage of cells that fall into that quadrant.

[3H]thymidine uptake increased in depleted cultures from the BLV-seronegative cow. CD8 T-lymphocyte-depleted control cultures showed no significant change in spontaneous proliferation (data not shown). When examined on an individual cow basis, the percent reduction (20–53%) in [3H]thymidine uptake following CD4 T lymphocyte depletion could not be totally accounted for by the percentage (3–24%) of live, blast-size T lymphocytes eliminated.

To determine whether T lymphocytes spontaneously incorporate [3H]thymidine or become blast-sized cells in the absence of B lymphocytes we evaluated the level of 72 h spontaneous proliferation of PBMC and B-lymphocyte-depleted PBMC from three PL and three NEG cows. As expected the 72 h mean c.p.m. [3H]thymidine incorporation of non-depleted PBMC from PL cattle was significantly higher than that of NEG cattle ($P < 0.005$, one-tailed $t$-test). The level of [3H]thymidine incorporation for B-lymphocyte-depleted PBMC for all six cows was low (range 320–1634 c.p.m.) and there was no significant difference between PL and NEG cattle for mean c.p.m. [3H]thymidine incorporation ($P = 0.23$, one-tailed $t$-test). The percentage of live, blast-sized T lymphocytes at 3 days was also low (3–6%) in B-lymphocyte-depleted PBMC and did not differ significantly between PL and NEG cattle.

The majority of bovine peripheral blood B lymphocytes spontaneously enter $G_{s}/M$ of the cell cycle during short-term culture regardless of BLV infection or PL status and independent of any accessory cells.

Next we evaluated lymphocyte subset marker expression and DNA content (propidium iodide staining) of cultured PBMC to determine the percentage of B and T lymphocytes entering $G_{s}/M$ of the cell cycle in relation to BLV infection and PL status. For clarity, the term ‘proliferating cells’ will be restricted to cells that take up exogenous nucleotides during...
Table 2. Effect of CD4 T lymphocyte depletion on [3H]thymidine uptake by 72 h cultured PBMC from BLV-infected PL cattle

<table>
<thead>
<tr>
<th>BLV status</th>
<th>Cow no.</th>
<th>Live, blast-size T cells (%)</th>
<th>Mean c.p.m. (SD)</th>
<th>CD4-depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEG</td>
<td>1529</td>
<td>ND*</td>
<td>1197 (349)</td>
<td>1954 (838)†</td>
</tr>
<tr>
<td>PL</td>
<td>1374</td>
<td>24</td>
<td>5263 (1084)</td>
<td>2448 (859)†</td>
</tr>
<tr>
<td>PL</td>
<td>1379</td>
<td>20</td>
<td>7871 (1604)</td>
<td>3841 (627)†</td>
</tr>
<tr>
<td>PL</td>
<td>1440</td>
<td>ND*,§</td>
<td>10094 (2465)</td>
<td>4954 (1161)‖</td>
</tr>
<tr>
<td>PL</td>
<td>1583</td>
<td>3</td>
<td>38217 (8393)</td>
<td>30639 (1522)†</td>
</tr>
</tbody>
</table>

Mean c.p.m. (SD) Reduction in c.p.m. (%)

<table>
<thead>
<tr>
<th></th>
<th>Mean c.p.m. (SD)</th>
<th>Reduction in c.p.m. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No depletion</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4-depleted</td>
<td></td>
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</tr>
</tbody>
</table>

* ND, Not determined; NS, no significant decrease.
† 0% CD4 cells present by flow cytometric analysis at the initiation and termination of 72 h culture.
‡ Non-depleted and CD4-depleted cultures are significantly different at P ≤ 0.05.
§ Although not determined for this experiment, the sample from cow 1440 shown in Table 1 was drawn 6 weeks after the sample shown in Table 2 and shows 7% CD4 blast-size cells at 72 h culture.
‖ 0% CD4 cells present by flow cytometric analysis at the initiation of 72 h culture, but no flow cytometric analysis was done at the termination of culture.

Table 3. Percentage of B and T lymphocytes in G2/M in cultured PBMC from BLV-infected PL cattle and controls

Status (sample no.) | Mean percentage of B lymphocytes in G2/M 0 h 24 h 72 h P value*  
|-------------------|-------------------------------------
| PL (5)            | 13 (4)† 57 (9) 79 (11) 0.0001       |
| NPL (3)           | 23 (4) 54 (1) 53 (7) 0.0003         |
| NEG (4–5)#        | 23 (9) 51 (9) 57 (16) 0.03          |
| P value*          | NS § NS 0.01                         |

Mean percentage of T lymphocytes in G2/M

Status (sample no.) | Mean percentage of T lymphocytes in G2/M 0 h 24 h 72 h P value*  
|-------------------|-------------------------------------
| PL (5)            | 20 (5)† 19 (4) 34 (14) 0.03         |
| NPL (3)           | 22 (7) 28 (5) 27 (3) NS              |
| NEG (4–5)#        | 20 (12) 25 (7) 27 (11) NS           |
| P value*          | NS § NS NS                            |

* P values were obtained by analysis of variance on the samples represented in each row and in each column.
† Standard deviations (given in brackets).
‡ Four cows were sampled at 24 h. Five cows were sampled at 0 h and 72 h.
§ NS, Not significant at the P ≤ 0.05 level.

PBMC from BLV-seronegative, BLV-infected NPL, and BLV-infected PL cattle were cultured without stimulation and the percentages of B and T lymphocytes with 4n DNA content were determined at 0, 24 and 72 h (Table 3). The 2% formaldehyde fixation that preceded permeabilization prevents leakage of fragmented DNA from the cell (Darzynkiewicz et al., 1995). PBMC from BLV-infected PL cattle showed that depletion of CD4, CD8 and γδ T lymphocytes with 4n DNA content in freshly isolated PBMC ranged from 13% to 23% but means did not differ significantly by BLV infection or PL status. By 24 h there was a significant increase from t = 0 in the percentage of B lymphocytes with G2/M DNA content in all cultures (P ≤ 0.001, calculation not shown in Table 3). The percentage of B lymphocytes in G2/M at 24 h was between 51% and 57% and did not differ significantly at P ≤ 0.05 by BLV infection or PL status. By 72 h the percentage of B lymphocytes in G2/M was significantly higher in PBMC cultures from PL cattle compared to cultures from BLV-infected NPL cattle or NEG cattle (P ≤ 0.01). When PBMC from four PL and three BLV-seronegative cattle were depleted of CD4 or CD8 T lymphocytes there was no significant change in the percentage of B lymphocytes in G2/M at 72 h (data not shown). Depletion experiments on PBMC from two PL cattle showed that depletion of CD2, CD4, CD8 and γδ T lymphocytes also had no effect, nor did the depletion of monocytes with and without T lymphocyte depletions (data not shown). In contrast to the movement of the majority of bovine B lymphocytes into G2/M, few if any T lymphocytes from NPL or BLV-seronegative cattle entered the cell cycle during the 72 h time-frame (Table 3). A low but
B lymphocytes became 4
not take up appreciable 
points (Fig. 2). Results demonstrated that bovine PBMC did 
and one BLV-seronegative cow at both the 24 h and 72 h time-
nucleotide uptake of unstimulated PBMC from two PL cattle 
positive for BrdU uptake by 24 h. To further evaluate this 
majority of bovine B lymphocytes into G2/M during spontaneous 
lymphocyte proliferation of PL cows; the p24-
expression is limited to a small 
percentage of B lymphocytes from PL cows; the p24-
lymphocyte proliferation of PL cows is, in part, mediated by 
percentage of B lymphocytes from PL cows that enter 
G2/M during spontaneous proliferation

The above results demonstrated that the majority of bovine B lymphocytes became 4n cells by 24 h of culture and yet few lymphocytes, B or T, from the one PL cow tested stained positive for BrdU uptake by 24 h. To further evaluate this unexpected observation we determined total exogenous nucleotide uptake of unstimulated PBMC from two PL cattle and one BLV-seronegative cow at both the 24 h and 72 h time-points (Fig. 2). Results demonstrated that bovine PBMC did not take up appreciable [3H]thymidine during the first 24 h of culture, whereas PBMC from PL cattle showed the expected marked [3H]thymidine uptake by 72 h. Thus, movement of the majority of bovine B lymphocytes into G2/M of the cell cycle by 24 h of culture regardless of BLV infection or PL status was not associated with appreciable [3H]thymidine uptake. However, the moderate increase in the percentage of B and T lymphocytes that entered G2/M between 24 and 72 h observed only in cells from PL cattle was associated with a marked increase in [3H]thymidine uptake.

In vitro BLV p24 expression is limited to a small percentage of B lymphocytes from PL cows; the p24-
expression of B lymphocytes are less likely to enter G2/M of the cell cycle compared to the overall progression of B lymphocytes into G2/M during spontaneous lymphocyte proliferation

Results from previous studies suggest that the spontaneous lymphocyte proliferation of PL cows is, in part, mediated by viral gene expression (Trueblood et al., 1998; Thorn et al., 1981). To investigate this question we determined the number and cell cycle position of lymphocytes that expressed viral proteins during culture by flow cytometric analysis of PBMC labelled intracellularly with anti-BLV p24 antibody. Because the vast majority of the circulating B lymphocytes from PL cattle contain provirus (Mirsky et al., 1996, 1993) and other PBMC subsets are rarely if ever infected (Mirsky et al., 1996), any p24-expressing cells were assumed to be B lymphocytes. Our results revealed that p24 expression was not detectable in freshly isolated or cultured PBMC from BLV-infected NPL cattle (data not shown). Although viral p24 expression was also not detectable in freshly isolated PBMC from PL cattle (data not shown), it was detectable in 5–15% of lymphocytes from four PL animals after 24–72 h culture, indicating that only a subset of B lymphocytes that contain provirus express BLV p24 during culture. Results from one PL cow (Fig. 3a) demonstrated that at 24 h the majority of all B lymphocytes were in G2/M of the cell cycle (76%), whereas only 16% of the p24-expressing lymphocytes were in G2/M. Next we evaluated cell cycle progression of p24-expressing cells from additional PL cattle over the time-frame of spontaneous lymphocyte proliferation and compared these data to cell cycle progression of B and T lymphocytes from PL cattle shown in Table 3 and collected as part of the same experiments (Fig. 3b). Results demonstrated that the majority of p24-expressing lymphocytes did not progress into G2/M during the time-frame of spontaneous lymphocyte proliferation. This was in sharp contrast to the early and marked overall progression of B lymphocytes into G2/M of the cell cycle (Fig. 3a, b). Cell size and cell granularity analysis confirmed that only a small percentage (mean of 11% and 14% at 24 h and 72 h respectively) of BLV p24-expressing lymphocytes were live, blast-size cells during spontaneous lymphocyte proliferation. To confirm by another method that the majority of proliferating cells were not virus-expressing cells, PBMC from one PL cow were cultured with BrdU. Dual staining for BLV expression and BrdU required a BLV antigen detectable on the cell surface. Therefore we stained for gp51 instead of the internal antigen p24 (Fig. 4). Results showed that few if any of the BrdU-incorporating lymphocytes displayed viral gp51 on the cell surface.

The majority of B lymphocytes from PL cows that enter G2/M of the cell cycle during spontaneous proliferation undergo apoptosis, whereas the virus-expressing B lymphocytes are more likely to remain in G0/G1, and are spared from apoptosis

We next investigated the possibility that the B lymphocytes entering G2/M of the cell cycle during culture are more likely to undergo apoptosis than the virus-expressing cells that remain in G0/G1. Experiments conducted on PBMC from three NEG cows showed that a mean of 72% (7% SD) of B lymphocytes were apoptotic by 24 h. Fig. 5 shows the results
BLV expression and B lymphocyte expansion

Fig. 3. (a) By 24 h post-culture the percentage of BLV p24-expressing lymphocytes in G2/M of the cell cycle is markedly less than the overall percentage of B lymphocytes in G2/M. The three histograms show the percentage of B lymphocytes at t = 0 and t = 24 h and p24-expressing lymphocytes at t = 24 h that are in G2/M of the cell cycle as measured by propidium iodide staining of permeabilized lymphocytes (FL2-H). The 2% formaldehyde fixation procedure used prior to permeabilization prevents leakage of small molecular mass DNA fragments from the nucleus so that total DNA content includes fragmented, apoptotic DNA. Similar results were obtained on cultured lymphocytes from four additional PL cows evaluated between 16 and 24 h. (b) The majority of BLV p24-expressing B lymphocytes from PL cows do not progress into G2/M of the cell cycle during the time-frame of spontaneous lymphocyte proliferation. Represented are the mean and SD of data from five PL cows showing the percentage of B, T and p24-expressing lymphocytes in G2/M, except for the p24 72 h time-point, which represents data from three of the five PL cows.

Fig. 4. Lymphocytes incorporating BrdU during spontaneous lymphocyte proliferation do not display viral gp51 on the cell surface. PBMC were surface stained by single fluorescence for BLV gp51-PE (FL2-H). Lymphocytes staining with anti-BrdU-FITC MAb were gated and are depicted in the histograms. The histograms show the percentage of BrdU-staining lymphocytes that express surface BLV gp51 at 48 and 72 h post-culture.

Fig. 5. (a) By 24 h cultured PBMC from a PL cow. Cultures were repeated on PBMC from two additional PL cows with similar results. Results showed that freshly isolated PBMC from PL cattle were not apoptotic (Fig. 5a), but after 24 h of culture 82% of B lymphocytes showed evidence of DNA fragmentation (Fig. 5b), similar to results of B lymphocytes from the three NEG cows. Measurements at 72 h showed only a slight increase in the percentage of apoptotic B lymphocytes over the 24 h time-point (data not shown). In sharp contrast to the high percentage of total B lymphocytes in apoptosis by 24 h, only 23% of p24-expressing lymphocytes were apoptotic at this time-point (Fig. 5c). Simultaneous flow cytometric analysis for stage of cell cycle and apoptosis revealed that 95% of the PBMC in G2/M were apoptotic and that 80% of the cells with less than 4n DNA content were not apoptotic by 24 h (Fig. 5d).

If viral protein expression promotes a delay in cell cycle progression and sparing from apoptosis, one would expect this pattern to be maintained even when the percentage of virus-expressing cells is increased by IL-2 stimulation (Trueblood et al., 1998). We evaluated cell cycle position and apoptosis of PBMC from a PL cow with and without 16 h IL-2 stimulation. IL-2 stimulation increased the number of virus-expressing lymphocytes from 10% to 17%. Regardless, the cell cycle and apoptosis profiles of the virus-expressing cells remained the same in that these cells were more likely to remain in G2/M and less likely to undergo apoptosis during short-term culture compared to B lymphocytes overall (data not shown).
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Discussion

In this study we established that the proliferating lymphocytes in spontaneous lymphocyte proliferation were not the virus-expressing lymphocytes. Whether the proliferating B lymphocytes are infected with BLV is not known. In agreement with a previous report (Dequiedt et al., 1999) we observed that lymphocytes expressing BLV p24 during unstimulated culture were spared from apoptosis. Our results extend this observation and suggest that the ability of BLV expression to prevent apoptosis may be tied to its ability to arrest or delay cells in G₂/M of the cell cycle. A number of in vitro mechanisms used to arrest cells at the G₂ checkpoint can prevent cell death (Bissonnette & Hunting, 1998; Gorospe et al., 1997). For example, G₂₁ cell cycle arrest induced by expression of the cyclin-dependent kinase inhibitor p21 can prevent apoptosis following DNA damage (Bissonnette & Hunting, 1998). Cell cycle arrest by a retroviral protein has been demonstrated for the human immunodeficiency virus (HIV) type 1 Vpr protein. Although Vpr expression causes a G₂ arrest and not a G₁ arrest (Jowett et al., 1995; He et al., 1995; Bartz et al., 1996), the cell cycle arrest is associated with increased virus expression (Goh et al., 1998; Yao et al., 1998). Furthermore, Vpr expression can disrupt T-cell-receptor-mediated induction of apoptosis (Ayyavoo et al., 1997). In HTLV-I, considerable attention has focused on the anti-apoptotic and cell cycle regulatory properties of the transactivating protein Tax. Both HTLV-I-expressing T lymphocyte lines and uninfected, primary human T lymphocytes treated with soluble Tax are less susceptible to Fas-mediated apoptosis (Copeland et al., 1994). Tax expression has been associated with altered expression of some cyclins and cyclin-dependent kinase inhibitors but, in general, HTLV-I Tax induces alterations favouring proliferation and not cell cycle arrest (Akagi et al., 1996). Thus, BLV may be unique among retroviruses in its association with G₂/M delay or arrest, but share similarities with HTLV in its ability to increase the survival of virus-expressing cells.

Because suppression of BLV expression results in decreased spontaneous lymphocyte proliferation (Trueblood et al., 1998; Jensen et al., 1992), our results suggest that viral expression by a subset of B lymphocytes promotes the proliferation of B lymphocytes that do not express virus. One possibility is that secreted viral proteins activate T lymphocytes, cells not infected by BLV, that in turn promote B lymphocyte proliferation. This is consistent with previous reports that spontaneous lymphocyte proliferation is, in part, mediated by...
IL-2 (Trueblood et al., 1998; Stone et al., 1994, 1995) and our observations that a subset of CD4 T lymphocytes proliferate during spontaneous proliferation, that CD4 T lymphocyte depletion significantly decreases spontaneous proliferation, and that T lymphocytes in the absence of B lymphocytes do not become blast-size or take up appreciable $[^{3}H]$thymidine. Other retroviruses code for proteins with important biological effects on uninfected T lymphocytes (Marriott et al., 1991, 1992; Lindholm et al., 1990; Li et al., 1995, 1997; Ensoli et al., 1993, 1990; Mann & Frankel, 1991). In HTLV, the trans-activating protein Tax is secreted (Lindholm et al., 1990) and can activate uninfected T lymphocytes (Marriott et al., 1991). In HIV, the transactivating protein Tat can activate uninfected, quiescent T lymphocytes, generating a population of cells more permissive to infection (Li et al., 1997).

B lymphocyte proliferation in BLV may also be due to a direct effect of secreted viral proteins on B lymphocytes. Our observation that CD4 T lymphocyte depletion decreases but does not abolish spontaneous lymphocyte proliferation and a previous report that purified B lymphocytes from PL cattle also spontaneously proliferate (Trueblood et al., 1998) lend support to this possibility.

Results from this study also revealed that the majority of both normal bovine B lymphocytes and B lymphocytes from BLV-infected cattle spontaneously enter G$_{2}$/M of the cell cycle and die by apoptosis within the first 24 h of unstimulated culture. Of interest was the lack of association between this early B lymphocyte entry into G$_{2}$/M and the number of BrdU-staining cells or the amount of $[^{3}H]$thymidine uptake. One possible explanation is that bovine B lymphocytes initially utilize internal stores of nucleotides for the salvage pathway of DNA synthesis. Alternatively or additionally, bovine B lymphocytes may utilize the de novo pathway of DNA synthesis when first put into culture.

Our data provide evidence that BLV expression increases survival of the virus-expressing cells themselves and may promote the proliferation of virus non-expressing B lymphocytes. If the low level but persistent virus expression that is known to occur in vivo in BLV-infected cattle is similarly able to perturb B lymphocyte cell cycle progression, survival and proliferation, this would greatly promote virus expansion within the host. First, increased survival of the non-dividing, virus-producing cells would maximize virus production. Second, if BLV more efficiently infects and integrates into dividing cells as has been shown for other retroviruses (reviewed by Luciw & Leung, 1994) then virus-induced proliferation of uninfected B lymphocytes would expand the number of target cells for infection. Over time these processes would result in B lymphocyte expansion and increased numbers of virus-infected cells, the defining characteristics of BLV-infected PL cattle. Because PL cattle are the main source of transmission of virus to uninfected animals (Hopkins & DiGiacomo, 1997; DiGiacomo, 1992), these processes would promote spread of the virus within the population. BLV infection of an outbred population of animals under natural conditions offers an excellent animal model system to investigate the effects of virus expression on lymphocyte activation and proliferation. In particular, our results provide evidence that BLV, like HTLV and HIV, may have important biological functions on uninfected T lymphocytes that may promote virus spread and disease progression.

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