Reduced IL-2 and IL-4 mRNA Expression in CD4+ T Cells from Bovine Leukemia Virus-Infected Cows with Persistent Lymphocytosis

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The role of T-helper (Th) responses in the subclinical progression of bovine leukemia virus (BLV) infection was explored by determining the contribution of CD4+ T cells to the expression of mRNAs encoding interferon-γ (IFN-γ), interleukin-2 (IL-2), interleukin-4 (IL-4), and interleukin-10 (IL-10) in BLV-infected cattle. Relative levels of mRNA encoding IFN-γ, IL-2, IL-4, and IL-10 were measured in fresh and concanavalin A (Con A) activated peripheral blood mononuclear cells (PBMCs) and purified CD4+ T cells from cows seronegative to BLV (BLV−), seropositive without persistent lymphocytosis (BLV+ PL−), and seropositive with PL (BLV+ PL+) using a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. The expressions of IFN-γ, IL-2, and IL-4 mRNAs were significantly reduced in the PBMCs from BLV+ PL− cows as compared to BLV− cows. Reduced levels of IL-2 and IL-4 mRNAs were detected in fresh CD4+ T cells from BLV+ PL− cows. In contrast, Con A-stimulated PBMCs and CD4+ T cells did not differ significantly in expression of IFN-γ, IL-2, IL-10, or IL-4 mRNAs among the BLV infection groups. Using flow-sorted CD4+ T cells and semiquantitative RT-PCR the frequencies of CD4+ T cells transcribing IFN-γ, IL-2, IL-4, and IL-10 mRNAs in the peripheral blood of BLV−, BLV+ PL−, and BLV+ PL+ cows were determined. There were no significant differences in the frequencies of CD4+ T cells expressing these cytokine mRNAs among animals in the different BLV infection categories. Thus, the observed differences in IL-2 and IL-4 mRNAs in CD4+ T cells were due to changes in steady-state mRNA levels expressed by individual cells and not to changes in the frequency of cells transcribing IL-2 and IL-4 mRNAs. These results demonstrate that the progression of BLV infection to PL is associated with reduced expression of classical Th1 and Th2 cytokines by CD4+ T cells, thus suggesting aberrant Th regulation in subclinically infected animals. © 2002 Elsevier Science (USA)

INTRODUCTION

The bovine leukemia virus (BLV) is a delta-retrovirus, a taxonomic group that includes the human T cell lymphotropic viruses (HTLVs), the simian T cell lymphotropic viruses, and the baboon T cell leukemia virus. These viruses share many common features of molecular organization and pathogenesis, including multiple provirus integration sites in host DNA, transcriptional repression of the provirus in vivo, chronic transforming potential, persistence of infection despite a strong humoral immune response, and resistance to progression of disease that is under genetic control of the host (Sagata et al., 1985; Jensen et al., 1991; Xu et al., 1993; Kettmann et al., 1994). A major difference between BLV and other known delta-retroviruses is that B lymphocytes are the primary target of BLV infection. Approximately 30% of infected cows develop an advanced subclinical stage of infection termed persistent lymphocytosis (PL) that is characterized by polyclonal expansion of the CD5+ B cell subset, the majority of which harbors BLV provirus (Kenyon and Piper, 1977; Mirsky et al., 1996). A small fraction of BLV-infected cows (1–10%) develop lymphoma, a fatal neoplastic disease that may involve lymph nodes, abomasum, uterus, and other tissues (Ferrer et al., 1979). Recent evidence suggests that inhibition of DNA repair enzymes by BLV-tax may be a necessary first step in the transformation process (Philpott and Buehring, 1999).

Both genetic and immunological factors contribute to susceptibility to PL and lymphosarcoma (reviewed in Lewin et al., 1999). For example, the presence of glutamic acid and arginine at positions 70–71 (peptide-binding residues) of the β1 domain of BoLA-DRB3 major histocompatibility complex (MHC) class II alleles is strongly associated with resistance to PL (Xu et al., 1993) and the spread of BLV in vivo (Mirsyk et al., 1998). Similarly, resistance to the development of lymphoma in sheep was associated with residues 70–71 of the major expressed sheep DRβ molecule (Nagaoka et al., 1999). Resistance in sheep was also correlated with T cell proliferative responses to epitopes on the major BLV envelope glycoprotein gp51 (Nagaoka et al., 1999). Multiple lines of evidence suggest that cell-mediated immunity plays an important role in limiting the spread of BLV in vivo, consistent with MHC class II involvement in disease progression. Strong T cell proliferative responses to the major core protein (p24) and the major...
envelope glycoprotein (gp51) were demonstrated in cattle with PL (Callebaut et al., 1993; Mager et al., 1994) and in sheep (Gatei et al., 1993a). These responses appear to be driven mainly by the CD4\(^+\) T cell subset (Callebaut et al., 1993; Gatei et al., 1993b; Mager et al., 1994).

The demonstrable cell-mediated immune responses to BLV epitopes, the significant changes in frequency of B cell subsets, and the immunological aberrations observed in PL cattle have led several investigators to examine the expression of cytokines or cytokine mRNAs in animals with advanced subclinical and clinical stages of BLV infection. Diminished expression of IFN-\(\gamma\), IL-2, and IL-12 p40 mRNAs and increased IL-10 mRNA were found in freshly isolated PBMCs from BLV\(^+\) PL\(^-\) and tumor-bearing animals, thus suggesting a T-helper-2 (Th2) response shift associated with PL (Pyeon et al., 1996; Pyeon and Splitter, 1998). In support of these findings, Keefe et al. (1997b) found that interferon-\(\gamma\) (IFN-\(\gamma\)) and interleukin-2 (IL-2) mRNAs were reduced in T cells isolated from the lymph nodes of cattle with PL. Although these results suggest that the potential for cytokine production differs in PBMCs from cows with PL and tumors, Pyeon and co-workers (1996, 1998) did not control for the number of T and B cells used for mRNA extractions. Thus, it is unclear whether the observed changes in cytokine mRNA expression were due to differences in the frequency of T cells or other lymphoid subsets that are altered as a result of BLV infection. In the present study, we directly assessed the contribution of the CD4\(^+\) T cell subset to IFN-\(\gamma\), IL-2, IL-4, and IL-10 mRNA expression in BLV-infected cattle. In addition, the frequencies of fresh peripheral blood CD4\(^+\) T cells transcribing IFN-\(\gamma\), IL-2, IL-4, and IL-10 mRNAs were determined to understand whether differences in transcript levels are due to the changes in the numbers of cells expressing each cytokine mRNA or to changes in level of expression within the total CD4\(^+\) T cell pool.

**RESULTS**

**Cytokine mRNA expression in PBMCs**

A semiquantitative RT-PCR assay was used to compare the levels of cytokine mRNAs expressed by fresh and concanavalin A (Con A) activated peripheral blood mononuclear cells (PBMCs) from BLV\(^-\), BLV\(^+\) PL\(^-\), and BLV\(^+\) PL\(^+\) cows. Reduced levels of IFN-\(\gamma\), IL-2, and IL-4 mRNAs were found in PBMC from BLV\(^+\) PL\(^+\) cows when compared to BLV\(^-\) animals \((P < 0.05, \text{Fig. } 1)\). Fresh PBMCs from BLV\(^+\) PL\(^-\) cows transcribed less IFN-\(\gamma\) \((P < 0.06)\) and IL-4 \((P < 0.05)\) mRNA than did BLV\(^-\) cows (Fig. 1). The only significant comparison \((P < 0.05)\) between BLV\(^+\) PL\(^+\) and BLV\(^+\) PL\(^-\) animals was for IL-2 mRNA, which was significantly lower in PBMCs among cows with PL, although expression of IL-10 mRNA also tended to be reduced \((P < 0.1)\). When PBMCs were cultured for 24 h with Con A, there were no significant differences in expression of cytokine mRNAs among the three groups of cows (Fig. 2).
Cytokine mRNA expression in CD4⁺ T cells

The expressions of cytokine mRNAs were compared in immunomagnetic bead-purified CD4⁺ T cells. Reduced expression of IL-2 and IL-4 mRNAs was observed in fresh CD4⁺ T cells from cows with PL as compared to BLV⁻ animals (P < 0.05), whereas the levels of IFN-γ and IL-10 mRNAs were similar among the BLV infection groups (Fig. 3). In addition, fresh, purified CD4⁺ T cells from animals with PL expressed less IL-2 mRNA than BLV⁻PL⁻ animals. Among the cows with PL, IL-2, and IL-4 mRNA levels were approximately four- and threefold greater in purified fresh CD4⁺ T cells than in fresh PBMCs, respectively (compare Figs. 1 and 3). Similar results were observed for both IL-2 and IL-4 in BLV⁺PL⁻ and BLV⁻ animals, demonstrating that CD4⁺ T cells are a major source of IL-2 and IL-4 in cattle. In contrast, the levels of IL-10 mRNA were not different in fresh CD4⁺ T cells compared to fresh PBMCs from cows with PL, although Con A stimulation of PBMC and CD4⁺ T cells from cows with PL produced approximately a twofold increase in IL-10 mRNA relative to fresh PBMC and fresh CD4⁺ T cells from PL cows. Thus, although IL-10 is known to be produced primarily by monocyte/macrophage in cattle (Pyeon et al., 1996), IL-10 may also be produced by CD4⁺ T cells. The activation of CD4⁺ T cells from BLV⁺PL⁺ cows with Con A resulted in a marked increase in the levels of IL-2 and IL-4 mRNA, reaching values comparable to BLV⁻ and BLV⁺PL⁻ animals (Fig. 4).

Frequencies of CD4⁺ T cells transcribing cytokines in peripheral blood

Preliminary cell-sorting and cell-frequency analysis revealed that less than 1% of fresh CD4⁺ T cells expressed any given cytokine mRNA (data not shown). Therefore, a multicell sorting approach was used to determine the relative frequencies of CD4⁺ T cells transcribing IFN-γ, IL-2, IL-4, and IL-10 mRNAs. Among the infection groups, there was no significant difference in the frequencies of CD4⁺ T cells transcribing any of the cytokine mRNAs tested (Table 1). The frequency of fresh CD4⁺ T cells transcribing IL-2 ranged from 2.7- to 4.7-fold greater than the frequency of CD4⁺ T cells transcribing IFN-γ, depending on BLV infection group. These results demonstrate that the transcription of the Th1 cytokines IL-2 and IFN-γ is regulated independently in cattle. Similarly, the frequency of CD4⁺ T cells transcribing IL-10 was up to 3.6-fold higher than the frequency of CD4⁺ T cells transcribing IL-4, demonstrating that the transcription of these cytokines is decoupled in cattle. Thus, differences in the levels of cytokine mRNA expression found in fresh cells among animals grouped by infection status are likely to be due to differences in steady-state mRNA levels.
FIG. 3. Mean cytokine mRNA expression in fresh CD4⁺ T cells from BLV⁻ (n = 8), BLV⁺PL⁻ (n = 8), and BLV⁺PL⁺ cows (n = 9). Means are expressed as the ratio of the cytokine/GAPDH IDV. Error bars denote standard errors. Means with different letters are different (P < 0.05).

FIG. 4. Mean cytokine mRNA expression in Con A activated CD4⁺ T cells from BLV⁻ (n = 8), BLV⁺PL⁻ (n = 8), and BLV⁺PL⁺ cows (n = 9). Means are expressed as the ratio of the cytokine/GAPDH IDV. Error bars denote standard errors.
PBMC not to be different in BLV ours in that we found the levels of IL-10 mRNA in fresh The results of Pyeon and co-workers are at odds with by inducing a Th2 polarization of the immune response. IL-10 plays a key role in the progression of the disease state mRNA levels within individual CD4T cells, not the number of transcriptionally active cells.

**DISCUSSION**

Levels of IFN-γ, IL-2, IL-4, and IL-10 transcripts in fresh PBMCs and purified CD4 T cells from BLV-, BLV+PL-, and BLV+PL+ cows were studied to determine whether BLV infection and disease progression are associated with perturbations in Th cytokine mRNA expression profiles. Cytokines were chosen to represent classical Th1- and Th2-like mediators. In addition, we estimated the fractions of cells expressing these cytokine mRNAs to determine whether differences in transcript levels among the groups were due to changes in the steady-state levels of mRNA on an individual cell basis or to increases in the frequencies of cells expressing the different cytokine mRNAs. Previously, Pyeon et al. (1996) showed that PBMCs from BLV+PL- cows transcribe significantly less IFN-γ and IL-2 mRNAs than BLV+PL- cows and that PBMCs from BLV+PL+ cows transcribe much higher levels of IL-10 mRNA than the PBMCs from BLV+PL- and BLV+ cows. These authors suggested that IL-10 plays a key role in the progression of the disease by inducing a Th2 polarization of the immune response. The results of Pyeon and co-workers are at odds with ours in that we found the levels of IL-10 mRNA in fresh PBMC not to be different in BLV+PL- and BLV+ cows. In addition, BLV+PL- cows’ PBMCs tended to have increased IL-10 mRNA levels relative to both BLV- and BLV+PL- cows in our study. Although these discrepancies may be attributed to differences in technical approach (see below), it is not likely that a Th2 shift causes PL because there is no convincing evidence that IL-10 behaves as a Th2 cytokine in cattle (Brown et al., 1993, 1994a,b, 1998). In addition, we found IL-4 mRNA expression in the CD4 T cells of all tested animals, with expression levels significantly greater in uninfected animals than in infected animals. Thus, results obtained for IL-10 and IL-4 (Romagnani, 1997) do not support the idea that a positive Th2 shift is a key factor in the polyclonal expansion of BLV-infected B cells observed in animals with PL. We believe that other factors, such as MHC class II mediated effects on Th1 responses (Mirsy et al., 1998), enhanced clonal proliferation of BLV-infected B cell precursors, or resistance to apoptosis (Cantor et al., 2000), are more likely to be responsible for the development and maintenance of the PL state. The reason for the decreased IL-4 mRNA levels in cows with PL and its immunological significance remains to be elucidated.

The numbers of T cells used in the assays were not standardized in previous studies of cytokine mRNA expression and BLV infection (Keefe et al., 1997a; Trueblood et al., 1998; Pyeon and Splitter, 1999). Cattle with PL will often have 25% the normal percentage of T cells in peripheral blood (Mirsy et al., 1996). This change is directly attributable to the polyclonal expansion of BLV-infected CD5+ B cells in BLV-infected animals (Mirsy et al., 1998). Therefore, it is difficult to draw meaningful conclusions from earlier studies in which a fixed number of fresh or cultured PBMC were used for mRNA extraction because the absolute number of T cells in such cultures may vary fourfold among infected and uninfected animals. The present study examined both PBMC and CD4+ T cells for cytokine mRNA levels, thus allowing comparison with results of previous studies and direct comparisons of transcript levels in equal numbers of CD4+ T cells. We observed a significant reduction in IL-2 and IL-4 mRNA in both fresh PBMC and purified CD4+ T cells in cattle with PL. These results are consistent with those of Keefe et al. (1997a,b), who found decreased levels of IL-2 mRNA and no change in IL-10 mRNA in lymph node T cells from cattle with PL (IL-4 was not measured). In addition, these results support earlier findings demonstrating that CD4+ peripheral T cells are a primary source of IL-2 and IL-4 transcripts. The high percentage of B cells in the preparations would not affect the IL-2 and IL-4 mRNA content of the fresh PBMC and PBMC cultures because these cytokines are not produced by cattle B cells. Although T cell percentage and absolute numbers could account for the differences observed in IL-2 and IL-4 mRNA levels among infection groups, this would not be a factor in the purified CD4+ T cells. In contrast, the lower expression of IFN-γ mRNA in fresh PBMC as compared to fresh CD4+ T cells in PL cattle (Figs. 1 and 3) suggests either an effect of T cell concentration or another source of IFN-γ mRNA in the peripheral T cell pool exists which is deficient in its ability to produce IFN-γ mRNA. The latter explanation is plausible because CD8+ T cells (Rhodes et al., 1999) and γδ T cells (Rhodes et al., 2001) have been shown to be a major source of IFN-γ mRNA expression in cattle.

There was no correlation between the frequency of IL-2 and IL-4 mRNA levels and the frequency of unstimulated CD4+ T cells transcribing these cytokine mRNAs.

### Table 1

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>BLV- (n = 5)</th>
<th>BLV+PL- (n = 6)</th>
<th>BLV+PL+ (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>0.089 ± 0.016</td>
<td>0.137 ± 0.021</td>
<td>0.132 ± 0.031</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.414 ± 0.138</td>
<td>0.366 ± 0.093</td>
<td>0.378 ± 0.135</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.028 ± 0.008</td>
<td>0.039 ± 0.006</td>
<td>0.065 ± 0.010</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.058 ± 0.023</td>
<td>0.142 ± 0.064</td>
<td>0.142 ± 0.040</td>
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</tbody>
</table>

*PMBCs were labeled with anti-CD4 and sorted into microtiter plates (100 or 500 cells/well). RT-PCR was performed as described under Materials and Methods. An algorithm was used to convert the percentages of positive and negative wells into cell frequencies expressing each cytokine, as described by Mirsky et al. (1993). Results are expressed as mean frequencies (%) ± standard errors.
These findings suggest that the decrease in IL-2 and IL-4 mRNA expression in CD4⁺ T results from a reduced steady-state mRNA level on a single-cell basis and not from a reduced frequency of CD4⁺ T cells producing these cytokine mRNAs. It is clear from the frequency analysis that cellular expression of the cytokine mRNAs only partially overlaps, thus demonstrating that there is no clear-cut Th1 or Th2 phenotype in cattle. It is relevant that CD4⁺ T cell clones derived from cattle infected with Babesia bovis, Borrelia bigemina, or Fasciola hepatica can simultaneously transcribe the IFNG and IL4 genes (Brown et al., 1998), clearly supporting our results obtained with purified CD4⁺ T cells. Thus, we did not find any evidence at the level of cell-frequency analysis for the clonal expansion of Th2 effectors in cows with PL, a result that agrees well with our semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Mitogen-stimulated PBMCs from cows with PL have been reported to overexpress IL-2 and IL-10 and to down-regulate expression of IL-12 p40 (Keefe et al., 1997a,b; Trueblood et al., 1998; Pyeon and Splitter, 1999). Our analysis of IL-2, IL-4, IL-10, or IFN-γ transcript levels in Con A activated PBMCs did not reveal any significant differences among BLV⁺, BLV⁺/PL⁺, and BLV⁺/PL⁻ cows, although activated PMBCs from BLV⁺/PL⁺ cows tended to express less IL-2 than BLV⁻ and BLV⁺/PL⁻ cows. This was in marked contrast to the results obtained with fresh PBMC and CD4⁺ T cells from PL cows that expressed reduced levels of IL-2 and IL-4 mRNA. These results indicate that CD4⁺ T cells from PL cows are able to mount vigorous IL-2 and IL-4 responses when polyclonally activated. Moreover, the very large increase in IL-2 expression by Con A stimulated CD4⁺ T cells from PL cows as compared with unstimulated cells is consistent with the results of others (Stone et al., 2000). Although the mechanism of IL-2 hyperresponsiveness of T cells from PL cattle in vitro is unclear, it has recently been shown to be associated with the expression of BLV by infected B cells (Stone et al., 2000; J. Norimine and H. A. Lewin, unpublished observations). It was proposed that up-regulation of IL-2 may be caused by the transactivation of the IL2 gene in T cells by a soluble BLV protein (Trueblood et al., 1998). Alternatively, we favor the hypothesis that the expression of BLV antigens by infected B cells in vitro may lead to a polyclonal T cell activation. Recombinant IL-2 increases the expression of the IL-2 receptor α-chain and the BLV p24 protein in B cells isolated from BLV⁺/PL⁺ cows, supporting the existence of a positive feedback loop between IL-2 and viral expression (Stone et al., 1995; Trueblood et al., 1998).

Although BLV-infected cows do not show clear signs of immunosuppression, there is evidence indicating that their life span is shorter and that their productive performance is impaired (Wu et al., 1989; Brenner et al., 1989; Da et al., 1993). Moreover, immune dysfunctions have been reported in cows with PL, such as a decrease in the cytotoxic activity of CD8⁺ T cells (Yamamoto et al., 1984), the polyclonal expansion of CD6+ B cells (Mirskey et al., 1996), and a reduction in the phagocytic ability of monocytes or macrophages (Werling et al., 1998). We have demonstrated that both Th1 (IL-2) and Th2 (IL-4) cytokine mRNA expression is reduced in the CD4⁺ T cell subset of BLV⁺/PL⁺ cows and that IFN-γ mRNA expression might also be down-regulated in the CD8⁺ T cell compartment. These findings, in conjunction with the observation that CD4⁺ T cells from cows with PL show a diminished proliferative response to BLV gag and env antigens (Orlik and Splitter, 1996), indicate that the functionality of the CD4⁺ T cell subset becomes gradually compromised as the disease progresses. The mechanism that results in the apparent down-regulation of IL-2 and IL-4 mRNA expression is at present unclear. A possible mechanism might involve anergy induced by chronic exposure to BLV antigens as has been observed for mice infected with murine leukemia viruses (Fitzpatrick et al., 1992, Muralidhar et al., 1996). Further experiments will be required to understand if aberrant cytokine responses are a direct cause or the consequence of the immunological abnormalities observed in BLV-infected cattle. Moreover, it will be important to determine how these changes relate to the genetic differences of cattle in susceptibility to PL (Xu et al., 1993).

### MATERIALS AND METHODS

#### Animals and BLV infection status

All cattle were reared and maintained at the University of Illinois dairy research facility. BLV infection status was determined by using agar gel immunodiffusion to test for antibodies to the major BLV envelope glycoprotein, gp51 (Leukassay B, SynBiotics, Kansas City, MO). Persistent lymphocytosis among infected cows was defined on the basis of a standard hematological key (Mammerickx et al., 1978). The definition of PL is based upon significantly elevated lymphocyte counts in consecutive blood samples collected at least 3 months apart (Mammerickx et al., 1978). Between seven and nine cows in each of three BLV infection categories were selected for study. These categories were BLV⁺ (seronegative), BLV⁺/PL⁻ (seropositive, not PL), and BLV⁺/PL⁺ (seropositive with PL).

#### Cell separation, CD4⁺ T cell isolation, and Con A stimulation

Peripheral blood was collected by jugular venipuncture into acid-citrate dextrose (Vacutainer, Beckton–Dickinson Co., Rutherford, NJ). Buffy coat was harvested following centrifugation at 1000 g for 10 min and diluted 1:1 in phosphate-buffered saline (PBS, pH 7.4) containing 0.6% sodium citrate (Sigma, St. Louis, MO). Diluted cells were layered over Histopaque-1077 (Sigma) and centri-
fuged at 1000 g for 20 min. Peripheral blood mononuclear cells were removed from the gradient interface and washed three times in ice-cold PBS at 400 g for 10 min. Viability was assessed on a hemocytometer using 0.2% trypan blue. CD4⁺ T cells were isolated using an immunomagnetic separation technique. Briefly, PBMCs were resuspended in ice-cold citrate–PBS to a concentration of 5 × 10⁹ cells/ml and incubated for 30 min with 10 μg/ml of monoclonal mouse anti-bovine CD4 (CACT138A, VMRD Inc., Pullman, WA). After three washings with chilled PBS, cells were resuspended in ice-cold PBS and incubated with 1 × 10⁴ magnetic beads (45 μm) conjugated to goat anti-mouse IgG (Dynal, Oslo, Norway). The bead-bound cells were harvested using a Dynal Magnetic Particle Concentrator (Dynal) and washed extensively before release of bound cells by repeated aspiration with a 1-ml tuberculin syringe. The purified population contained >90% CD4⁺ T cells as determined using flow cytometry.

The cytokine mRNA expression profiles of PBMCs and CD4⁺ T cells were studied before and after activation with Con A (Boehringer Mannheim, Indianapolis, IN). Aliquots of freshly isolated PBMCs and purified CD4⁺ T cells were resuspended in Trizol reagent (Gibco-BRL, Grand Island, NY) immediately after isolation. Additional aliquots were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Harlan, Indianapolis, IN) and Con A (10 μg/ml) (Boehringer Mannheim). Cells were cultured in flat-bottom 96-well plates (Fisher Scientific, Pittsburgh, PA) at a concentration of 5 × 10⁶ cells/well (37°C, 5% CO₂). To mimic the physiological process of T cell activation, CD4⁺ T cells were stimu-

Semiquantitative reverse transcription-polymerase chain reaction

Cells were suspended in 1 ml Trizol (Gibco-BRL) and total RNA was isolated according to the manufacturer’s instructions. Reverse transcription reactions were performed in a 50 μl containing RT buffer (25 mM Tris–HCl pH 8.3, 37.5 mM KCl, 1.5 mM MgCl₂), 1 mM dithiothreitol (DTT), 2 μM oligo(dT₁₆), 250 μM of each dNTP and 200 U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). The amount of RNA used as a template for cDNA synthesis was 1 μg for fresh PBMCs and CD4⁺ T cells and 0.5 μg for cultured cells. RNA was incubated at 65°C for 10 min and subsequently placed on ice for 5 min. Reactions were performed at 42°C for 1 h followed by an inactivation step at 94°C for 10 min. The efficiency

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence (5’-3’)</th>
<th>PCR product size</th>
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<tr>
<td>(1) IFN-5’</td>
<td>ATCAATACAGGATGATCC</td>
<td>338 bp (1 + 2)</td>
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<tr>
<td>(2) IFN-3’</td>
<td>ATCCATGCTCTTTGAGACCG</td>
<td>192 bp (1 + 3)</td>
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<tr>
<td>(3) IFN-3’in</td>
<td>CTATACCTGGACCTTATGATC</td>
<td>172 bp (4 + 6)</td>
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<tr>
<td>(4) IL2-5’</td>
<td>CAACCCTGTGTTCTAAGCC</td>
<td>356 bp (4 + 5)</td>
</tr>
<tr>
<td>(5) IL2-3’</td>
<td>GTCCTAGATCGTTAGTCCA</td>
<td>172 bp (4 + 6)</td>
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<tr>
<td>(6) IL2-3’in</td>
<td>ATGATTGATCTGAGAGATT</td>
<td>313 bp (7 + 8)</td>
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<td>(7) IL4-5’</td>
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<td>(8) IL4-3’</td>
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<td>(11) IL10-3’</td>
<td>CCAGGATGCTCAGCTTCCTT</td>
<td>223 bp (14 + 15)</td>
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<td>(12) IL10-3’in</td>
<td>GCCAACCCAGGTAAACCTTAA</td>
<td>231 bp (16 + 17)</td>
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Note. in denotes an internal primer.
10 min. If equal amounts of a given PCR product were loaded in the outer lanes of the gel, the IDV measurement was lower than if they were loaded in the central lanes. To correct for this source of intragel variation, we coloaded a 223-bp standard control (SC) in each lane. Thus, we first corrected the IDV values for gel-positional effect by using the SC as a reference [corrected cytokine IDV (A) = IDV/SC IDV; GAPDH IDV (B) = IDV/SC IDV]. Next, we normalized the corrected cytokine IDVs to the corrected GAPDH IDV (A/B = corrected cytokine IDV). This method ensures that variations in the intensity of the PCR products were due exclusively to differences in the initial amount of cDNA templates. Semiquantitative data were analyzed with a general linear models procedure (SAS version 6.11, Cary, NC) in which treatment effect was the disease category and the dependent variables were the corrected and normalized IDVs for the four cytokines (TRT = IFN-γ + IL-2 + IL-4 + IL-10). Differences in cytokine mRNA expression among disease categories were tested by analysis of variance (SAS). Each data set (fresh PBMCs, cultured PBMCs, fresh CD4+ T cells, and cultured CD4+ T cells) was analyzed independently.

Frequency analysis of cytokine-expressing CD4+ T cells

The frequencies of CD4+ T cells transcribing cytokine mRNAs were determined by flow cytometry combined with RT-PCR for detecting gene expression in single or multiple cells distributed in microtiter plates according to previously published methods (Mirskey et al., 1996; Gaynor et al., 1996). Briefly, PBMCs were labeled with the CACT138A anti-CD4 primary antibody (VMRD) and the phycoerythrin-conjugated anti-mouse IgG1 secondary antibody (Southern Biotechnology Inc., Birmingham, AL). An EPICS 751 flow cytometer (Coulter Electronics, Hialeah, FL) equipped with a deposition unit for single-cell antibody (Southern Biotechnology Inc., Birmingham, AL). Each one of the five different second-round PCRs contained 5 μl buffer (500 mM KCl, 100 mM Tris, 25 mM MgCl2), 100 μM dNTPs, 0.4 μM of each primer, and 1 U Taq polymerase in 50 μl. The second-round PCRs consisted of an initial denaturation step of 94°C for 4 min followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 30 s and a final extension step of 72°C for 5 min. The PCR products were electrophoresed in 6% non-denaturing polycrylamide minigels and stained with ethidium bromide. The percentages of cells transcribing cytokine mRNAs were determined using an algorithm that takes into account amplification efficiency (Mirskey et al., 1993). The method is accurate for wells containing one cell or many cells (Mirskey et al., 1993). Frequencies of cells transcribing cytokine mRNAs were compared among animals classified according to BLV infection status using analysis of variance (SAS).

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