Increased Interleukin-10 mRNA Expression in Tumor-Bearing or Persistently Lymphocytotic Animals Infected with Bovine Leukemia Virus

DOHUN PYEON,¹ KATHY L. O'REILLY,² AND GARY A. SPLITTER^{1*}

*Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706,¹ and
Department of Veterinary Microbiology and Parasitology, Louisiana State University, Baton Rouge, Louisiana 70803²*

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Interleukin-10 (IL-10), produced by Th2 helper T cells, B cells, and macrophages, can inhibit cytokine production by Th1 cells and enhance B-cell proliferation and differentiation. Here, we show that peripheral blood mononuclear cells (PBMCs) from bovine leukemia virus-infected animals with late-stage disease express considerably more IL-10 mRNA than animals that are not infected or that are in the early stages of disease. In contrast, the quantities of type 1 cytokines, IL-2 and gamma interferon, decrease with disease progression. In addition, we observed that IL-10 is expressed principally by monocytes/macrophages, not B lymphocytes, in persistently lymphocytotic animals. This observation supports a role for monocytes/macrophages in progression of bovine leukemia virus infection and, of importance, indicates that proliferating B cells are not the source of IL-10 expression. These findings suggest that IL-10 produced by monocytes/macrophages may influence the progression of bovine leukosis in animals that develop persistent lymphocytosis of B cells or B-cell lymphosarcoma.

Bovine leukemia virus (BLV) is a type C retrovirus infecting bovine B cells and causing enzootic bovine leukosis (26). The disease is divided into three stages: serologically positive, persistent lymphocytosis negative (BLV⁺PL⁻); serologically positive, persistent lymphocytosis positive (BLV⁺PL⁺); and tumor formation (Tumor⁺). Most infected animals never show outward signs of disease and are referred to as asymptomatic or aleukemic. Thirty to seventy percent of infected animals progress to BLV⁺PL⁺, in which nonneoplastic B cells proliferate and leukocyte counts may exceed 10,000 cells/mm³ (28), while only 10% of infected animals develop malignant lymphosarcoma (16). Usually a long duration is required between these disease stages, but the mechanisms of disease progression are not yet clear. Nevertheless, recently in human immunodeficiency virus (HIV) infection differences in cytokine production have been reported as the disease progresses (9). This finding suggests that cytokine imbalance may contribute to disease progression in AIDS and possibly other diseases, such as autoimmune disease and cancer (35). Interleukin-10 (IL-10) is an important cytokine influencing the balance between type 1 and type 2 cytokine profiles and is of potential importance in B-cell transformation (23). In this paper we demonstrate that IL-10 quantitatively increases through disease progression in BLV-infected animals, whereas IL-2 and gamma interferon (IFN- γ) decrease. In addition, we show that monocytes/macrophages are the principal source of the IL-10 increase.

Determination of disease stage and IL-10 production. The stage of BLV disease for animals was defined and confirmed by detection of antibodies to BLV by enzyme-linked immunosorbent assay (ELISA); B-cell numbers were determined by flow cytometry and cell counting. High antibody titers were detected with the sera of BLV-seropositive animals by ELISA, and animals with persistent lymphocytosis had 3,000 to 12,000

lymphocytes per mm³ of peripheral blood mononuclear cells (PBMCs). In PL⁻ animals, the proportion of B cells was 20 to 30% of the PBMCs and 55 to 70% of PBMCs were T cells. In contrast, PBMCs from PL⁺ animals had 57 to 80% B cells and only 10 to 30% T cells (data not shown).

Cytokine mRNA expression by animals in different disease stages was qualitatively detected by reverse transcriptase PCR. Interestingly, IL-10 message was detected from PBMCs of Tumor⁺ and BLV⁺PL⁺ animals but not from those of healthy or BLV⁺PL⁻ animals (Fig. 1). All tumor-bearing animals and four of five PL⁺ animals produced IL-10. In contrast, only two of four BLV⁺PL⁻ animals and no BLV⁻PL⁻ animals produced IL-10 (Table 1).

Quantification of cytokines produced by BLV-infected animals. To more critically assess cytokine levels produced by animals in different disease stages, we quantified a selected panel of cytokines by using mimics. β -Actin (5'-ACCAACTGGACGACATGGAG, 3'-GCATTTGCGGTGGACAATGGA; 890-bp product), IL-2 (5'-ACGGGGAACACAATGAAAGAAGT, 3'-CGTAGGGCTTACAAAAAGAATCT; 548-bp product), IL-4 (5'-CTATTAATGGGTCTCACCTACCA, 3'-CTTGCCAAGCTGTTGAGATTC; 311-bp product), IL-6 (5'-CCTTCACTCCATTGCTGTC, 3'-TTGCGTTCTTTACCCACTCG; 534-bp product), IL-10 (5'-GCCAAGCCTTGTCTGAGATGATC, 3'-CTCCCTGGTTTCTCTTCTCCTAAGA; 781-bp product), IFN- γ (5'-CTGTGGGCTTTTGGGTTTTCTG, 3'-CTCTTCCGCTTTCTGAGGTTAGA; 428-bp product), tumor necrosis factor alpha (TNF- α) (5'-CTGCACCTTCGGGTAATCGG, 3'-CAGGGCGATGATCCCAAAGTA; 549-bp product), and TNF- β (5'-AGACCCAGCAGCCAGGACTCG, 3'-GGAGATGCCATCTGTGTGAGTG; 361-bp product) were used as amplification primers (13). Amplified products were analyzed by 1% agarose gel electrophoresis. Cytokine mimics generated with PCR binding primers contain an internal non-specific stuffer fragment (12). The concentration of cytokine mimic-ligated plasmid was determined by spectrophotometry. Sensitivities of IL-2 and IL-4 mimics were 1 to 5 pg/ml, and sensitivities of IL-10 and IFN- γ mimics were approximately 1

* Corresponding author. Mailing address: Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, 1655 Linden Dr., Madison, WI 53706. Phone: (608) 262-1837. Fax: (608) 262-7420. Electronic mail address: gas@ahabs.wisc.edu.

FIG. 1. IL-10 expression by RT-PCR of BLV-infected animals at each disease stage. Total cDNA, 0.63 μ g and 3.15 μ g, was used as a template for β -actin and IL-10, respectively, to reflect the difference in β -actin and cytokine mRNA normally produced by cells. PCR products were analyzed on a 1% agarose gel. The product size was 890 bp for β -actin and 781 bp for IL-10, and the marker was a 1-kb ladder (Gibco BRL).

pg/ml and 5 to 10 pg/ml, respectively. Total RNA, including tRNA, rRNA, and mRNA, 4.32 to 36.42 μ g, was isolated from BLV-infected animals at different disease stages for IL-2 quantification, while 0.26 to 1.06 μ g of mRNA was isolated and tRNA and rRNA were removed for IL-4, IL-10, and IFN- γ quantification. Similarly, 0.01 to 0.05 μ g of cDNA from iso-

TABLE 1. IL-10 production in animals in different stages of bovine leukosis

Disease stage	No. of animals tested	No. of IL-10-positive animals
BLV ⁻ PL ⁻	8	0
BLV ⁺ PL ⁻	4	2
BLV ⁺ PL ⁺	5	4
Tumor ⁺	7	7

lated mRNA or 0.3 to 0.5 μ g of total cDNA was amplified with different concentrations of cytokine mimics (Fig. 2). Interestingly, IL-10 mRNA expression increased with progression of disease, whereas IL-2 and IFN- γ decreased (Fig. 3). In tumor-bearing animals, IL-10 production increased up to 30-fold compared with IL-10 production in seronegative animals. In contrast, IL-2 and IFN- γ mRNA expression decreased with progression of disease. IFN- γ production by tumor-bearing animals decreased up to 35-fold compared with BLV⁺PL⁻ animals, and IL-2 production was not detectable in tumor-bearing animals. BLV⁺PL⁻ animals had a 140-fold increase in IL-2 compared with BLV⁻ animals (Table 2).

We have shown that type 1 cytokines, IL-2 and IFN- γ , decreased with disease progression and that type 2 cytokines, IL-10, increased. However, we could not detect IL-4 mRNA expression at any stage of BLV infection, although IL-4 production was consistently detected in concanavalin A-stimulated PBMCs of noninfected animals (data not shown).

IL-10 is produced only by monocytes/macrophages. To determine which cells were producing IL-10, B lymphocytes were isolated with magnetic beads from PBMCs of BLV⁺PL⁺ animals with mouse anti-bovine immunoglobulin M (IgM) monoclonal antibody (no. 33, provided by K. Nielson, Animal Disease Research Institute, Nepean, Ontario, Canada) and

FIG. 2. Representative quantitative competitive-PCR bands of cytokines produced by BLV-infected animals at different disease stages. The same amount of total RNA was amplified with serially diluted cytokine mimics. Each cytokine mimic was used at the indicated concentration (picograms per milliliter) as a template with total mRNA for IL-10 and IFN- γ or with total RNA for IL-2 amplification. The sizes of products are as follows: IL-10, 781 bp; IL-10 mimic, 450 bp; IL-2, 548 bp; IL-2 mimic, 450 bp; IFN- γ , 426 bp; IFN- γ mimic, 400 bp. All products were analyzed on a 1% agarose gel stained with ethidium bromide.

FIG. 3. Changes in cytokine mRNA quantities expressed by BLV-infected animals at different disease stages. The values shown are the means of two to five different animals per group with the standard errors. The same amount of total RNA was amplified with serially diluted cytokine mimics, and bands obtained were analyzed with the computer software NIH Image 1.54. The concentration was 0.1 μ g of mRNA, for IL-10 and IFN- γ , or 1 μ g of total RNA, for IL-2.

confirmed by flow cytometry. Isolated B cells had 6.4% CD4⁺ T cells (IL-A12, provided by C. Baldwin, University of Massachusetts, Amherst) and 0.4% monocytes (monoclonal antibody CAM36A, Veterinary Medical Research and Development, Pullman, Wash.). Likewise, the B-cell-negative population had 1.9%, 52.7%, and 10.0% staining IgM⁺, CD4⁺ cells, and monocytes, respectively. Only the negatively selected cell population consisting of CD4⁺ cells and monocytes expressed IL-10 mRNA. Thus, we concluded that increased IL-10 mRNA expression by BLV⁺PL⁺ animals was not the product of B cells (Fig. 4).

Next, monocytes/macrophages were sorted from PBMCs of the animals described above with a monoclonal antibody (CAM36A) and confirmed by esterase staining (29). More than 90% of the positively selected cells exhibited dark brown α -naphthyl acetate staining, whereas cells from the negatively sorted portion stained yellow (data not shown). IL-10 mRNA expression was detected principally from positively sorted cells,

TABLE 2. Quantity of cytokine mRNA produced by BLV-infected animals

Disease stage	amol of cytokine mRNA produced		
	IL-10 ^a	IL-2 ^b	IFN- γ ^a
BLV ⁻ PL ⁻	16.8 $\pm$ 4.6	$\leq$ 8.5 ^c	$\leq$ 120.0 ^c
BLV ⁺ PL ⁻	72.9 $\pm$ 32.3	868.8 $\pm$ 311.0	3,635.0 $\pm$ 749.2
BLV ⁺ PL ⁺	150.5 $\pm$ 34.2	170.8 $\pm$ 31.7	853.3 $\pm$ 231.0
Tumor ⁺	230.4 $\pm$ 56.5	ND ^d	526.7 $\pm$ 184.6

^a The samples are 0.1 μ g of total mRNA isolated from fresh PBMC. Attomolar concentrations shown were calculated from picograms per milliliter of mimics.

^b The samples for IL-2 quantification were 1 μ g of total RNA isolated from fresh PBMC.

^c Sensitivities of cytokine mimics were as follows: IL-10 mimic, $\leq$ 12.2 amol; IL-2 mimic, $\leq$ 8.5 amol; IFN- γ mimic, $\leq$ 30.4 amol.

^d ND, not detected.

i.e., monocytes/macrophages, and minimal IL-10 mRNA expression was detected from other cells (Fig. 4). Therefore, we concluded that monocytes/macrophages are the main source of IL-10 mRNA expression by BLV⁺PL⁺ animals.

In the present study we demonstrated that IL-10 mRNA was produced in large, attomolar quantities by PBMCs of BLV-infected animals with progression of disease, whereas levels of type 1 cytokines, IL-2 and IFN- γ , were significantly reduced in BLV⁺PL⁺ and tumor-bearing animals. Although IL-4 produc-

FIG. 4. IL-10 expression by sorted B cells and monocytes/macrophages. B cells and monocytes/macrophages were separated with Dynabeads. Mouse anti-IgM and anti-bovine monocyte/macrophage IgG were used for B cells and monocytes/macrophages, respectively, followed by goat anti-mouse IgG-coated magnetic beads. Sorted B cells were confirmed by flow cytometry, and monocytes/macrophages were confirmed by esterase staining. Two different concentrations of sample total RNA, 0.9 μ g/ μ l and 0.3 μ g/ μ l, were amplified for detection of IL-10 expression. β -Actin was used as a positive control, and a reverse transcriptase (RT) reaction without RT (RT⁻) was performed for a negative control. The negative control is not shown on the gel of B-cell separation.

tion was not detected, these results showed a decrease in type 1 cytokines and an increase in IL-10. Detection of other cytokines, i.e., IL-6, TNF- α , and TNF- β , tested among animal groups with different stages of BLV infection was not associated with any disease stage (data not shown). IL-10 expression correlated with the more advanced stages of bovine leukosis, persistent lymphocytosis and tumor formation.

Similarly, high-level and continuous expression of IL-10 has been reported in the progression of AIDS (11), AIDS-related B-cell lymphoma (15), Burkitt's lymphoma caused by Epstein-Barr virus (EBV) (3), and non-Hodgkin's lymphoma (4). In a previous study by others, 8 of 15 patients with AIDS-related B-cell lymphoma produced large amounts of IL-10, whereas only 1 of 11 HIV-seronegative individuals produced IL-10 (15). Furthermore, 47% of patients with active non-Hodgkin's lymphoma produced IL-10, and only 5.8% of first remission patients and no healthy individuals produced IL-10 (4). These studies indicate the possibility that IL-10 contributes to disease progression and tumorigenesis.

We hypothesize that our IL-10 data support a role for this cytokine in influencing BLV disease progression leading to B-cell lymphosarcoma (23). Therefore, BLV in combination with IL-10 could have an important role in tumorigenesis as well as disease progression through three different pathways. First, IL-10, originally termed cytokine synthesis inhibitory factor (17), inhibits the expression of other cytokines, especially type 1 cytokines, e.g., IL-2 and IFN- γ . IL-10 also reduces expression of major histocompatibility complex class II molecules, mainly on macrophages, to inhibit antigen presentation (14). Inability of T cells to respond to presented foreign peptides could reduce IL-2, IL-6, and IFN- γ production. Second, IL-10 also has an ability to make Th1 cells undergo programmed cell death (8, 10). Th1 cells express high levels of Fas ligand and can be induced to programmed cell death, whereas Th2 cells express low levels of Fas and fail to undergo apoptosis (33). Reportedly, Fas antigen mediates Th1 cell activation in early in the immune response as well as apoptosis of Th1 cells late in the immune response (1), and only activated T cells can be killed by Fas-Fas ligand interaction (25, 30). Therefore, when BLV infects animals, Th1 cells may be activated, and later these activated Th1 cells would undergo programmed cell death. Thus, IL-2 and IFN- γ production might be reduced following apoptosis of Th1 cells. We suspect that this imbalance between type 1 and 2 cytokines may provoke the next disease stage of BLV infection (9). Many chronic diseases have a long asymptomatic period, as typified by HIV (18) and BLV (26) infection. In the present study, we have confirmed that cytokine synthesis changes with disease progression, resulting in cytokine imbalance in the late disease stages. Type 1 T-helper cells are an important part of the immune response against viral infection. Therefore, apoptosis of Th1 cells may cripple viral elimination as well as relatively increase the proportion of B cells. Third, IL-10 might contribute to persistent lymphocytosis and B-cell transformation in the late disease stages through viral proteins. In humans, IL-10 stimulates DNA replication of B cells following antigen receptor or CD40 cross-linking and enhances viability (6). A similar mechanism of B-cell activation could contribute to the lymphocytosis observed in cattle with leukosis. HIV Tat and gp120 induce IL-10 production through the activation of host transcription factors, including NF- κ B. Similarly, BLV encodes Tax and gp51 proteins, which are analogous at the genomic and functional level with HIV tat (31) and gp120 (2) proteins. In addition, an immunosuppressive peptide, termed CKS-17, possesses a common motif in retroviral transmembrane envelope proteins, including gp30 of BLV (20). This peptide can increase IL-10

production and suppress IL-2, IL-12, IFN- γ (21), and TNF- α production (19, 20) in human cell lines. Therefore, we hypothesize that IL-10 expression stimulated by BLV Tax, gp51, or gp30 protein induces proliferation and contributes to transformation of B cells. In BLV-infected cells, such as monocytes and B cells (34), tax and gp51 proteins are synthesized which may *trans*-activate or stimulate the IL-10 gene to express mRNA in monocytes/macrophages. In the genesis of Burkitt's lymphoma, EBV produces a viral IL-10-like molecule that induces proliferation and differentiation of B cells (24). Most B cells activated by EBV are normal B cells and secrete Ig. However, some EBV-infected B cells have a chromosomal translocation with juxtaposition of the oncogene *c-myc* into the Ig heavy chain or light chain genes (27). Dysregulation of *c-myc* gene expression may lead to B-cell transformation. Although not yet documented in BLV infection, IL-10 produced by macrophages may activate B cells and induce B-cell transformation by a similar mechanism. However, BLV infection can lead to B-cell differentiation and Ig class switching (22), supporting our speculation. In addition, the cytokine synthesis inhibitory factor function of IL-10 may inhibit the immune surveillance actions of T and NK cells, which are responsible for removing transformed cells (23). Thus, IL-10, stimulated by induced oncogenes, may lead to B-cell proliferation and suppression of tumor surveillance, resulting in tumor formation (23).

Moreover, we revealed that IL-10 expression of BLV⁺PL⁺ animals is due mainly to monocytes/macrophages in PBMCs. This result suggests a mechanism of disease progression and removes the possibility of IL-10 production by increased B cells. Macrophages are not only a prototypic antigen-presenting cell but also central in immune regulation secreting a variety of instructive cytokines, such as IL-1, IL-6, IL-8, IL-10, IL-12, IFN- γ , transforming growth factor β , and granulocyte-macrophage colony-stimulating factor (7). Recently, we found that indomethacin can reduce the amount of IL-10 mRNA produced by BLV⁺PL⁺ animals (32). This finding further substantiates the role of macrophages in IL-10 production, as indomethacin is a strong inhibitor of prostaglandin production by macrophages (5).

In summary, this study demonstrates that animals with lymphocytosis or lymphosarcoma in BLV infection produce increased amounts of IL-10, principally from monocytes/macrophages. These results suggest that monocytes/macrophages have an important role in disease progression of BLV-infected animals to the stage of persistent lymphocytosis and finally tumor formation. Further research linking prostaglandin and IL-10 production, as well as quantification of other cytokines, such as IL-12, produced by monocytes would aid in understanding the mechanism of cytokine imbalance mediated by monocytes/macrophages and BLV disease progression.

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