Prostaglandin E\textsubscript{2} Increases Bovine Leukemia Virus \textit{tax} and \textit{pol} mRNA Levels via Cyclooxygenase 2: Regulation by Interleukin-2, Interleukin-10, and Bovine Leukemia Virus

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Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), produced by macrophages, has important immune regulatory functions, suppressing a type 1 immune response and stimulating a type 2 immune response. Type 1 cytokines (interleukin-2 [IL-2], IL-12, and gamma interferon) increase in freshly isolated peripheral blood mononuclear cells (PBMCs) of animals with an early disease stage of bovine leukemia virus (BLV) infection, while IL-10 increases in animals with a late disease stage. Although IL-10 has an immunosuppressive role in the host immune system, IL-10 also inhibits BLV \textit{tax} and \textit{pol} mRNA levels in vitro. In contrast, IL-2 stimulates BLV \textit{tax} and \textit{pol} mRNA and p24 protein expression in cultured PBMCs. The inhibitory effect of IL-10 on BLV expression depends on soluble factors secreted by macrophages. Thus, we hypothesized that PGE\textsubscript{2}, a cyclooxygenase 2 (COX-2) product of macrophages, may regulate BLV expression. Here, we show that the level of COX-2 mRNA was decreased in PBMCs treated with IL-10, while IL-2 enhanced the level of COX-2 mRNA. Addition of PGE\textsubscript{2} stimulated BLV \textit{tax} and \textit{pol} mRNA levels and reversed the IL-10 inhibition of BLV mRNA. In addition, the specific COX-2 inhibitor, NS-398, inhibited the amount of BLV mRNA detected. Addition of PGE\textsubscript{2} increased BLV \textit{tax} mRNA regardless of NS-398 addition. PGE\textsubscript{2} inhibited antigen-specific macrophage stimulation, suggesting that stimulation of BLV \textit{tax} and \textit{pol} mRNA levels by PGE\textsubscript{2} is independent of cell proliferation. These findings suggest that macrophage-derived COX-2 products, such as PGE\textsubscript{2}, regulate virus expression and disease progression in BLV infection.

Bovine leukemia virus (BLV), closely related to human T-cell leukemia virus type 1 (HTLV-1), is a type C retrovirus that infects bovine B cells and leads to enzootic bovine leukosis (16). The genomes of BLV and HTLV-1 are similarly arranged. In particular, the 3' region that contains the \textit{tax}, \textit{rex}, R3, and G4 genes is unique to BLV and HTLV-1 (2). In addition, there are several features of pathogenesis that are shared by BLV and HTLV-1. For both viruses, many infected individuals develop antibodies, but clinical symptoms are relatively rare. Disease progression in BLV-infected animals is divided into three stages: serologically positive, persistent lymphocytosis negative (alymphocytotic [AL]); serologically positive, persistent lymphocytosis positive (persistently lymphocytotic [PL]); and tumor-bearing stages (usually lymphosarcoma). Most infected animals never display outward signs of disease and are referred to as asymptomatic or aleukemic. Fewer than 5% of infected animals develop malignant lymphosarcoma (11), while 30% of infected animals progress to persistent lymphocytosis, in which nonneoplastic B cells proliferate and leukocyte counts may exceed 10,000 cells/mm\textsuperscript{3} (17). Usually there is a long duration between these disease stages. The mechanism of disease progression from AL to PL or tumor-bearing stage is not clear.

Recent investigation has revealed that cytokine production plays a critical role in the progression of many different diseases (9, 32). In previous studies, we found cytokine polariza-

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follicular dendritic cells. The functions of COX-2 and prosta
glandins are very important in regulating normal physiological
processes (8, 19), as well as the immune response. Here, we
demonstrate that IL-10 decreased detection of COX-2 mRNA
by PBMCs, while conversely, IL-2 increased COX-2 mRNA.
Although PGE2 reduced antigen-specific PBMC proliferation,
PGE2 increased detection of BLV and reversed the IL-10
inhibition of BLV tax and pol mRNA levels. In addition, BLV
acts as an autocrine stimulator to increase the levels of BLV tax
and pol mRNA and COX-2 mRNA.

COX-2 mRNA from PBMCs was inhibited by IL-10 and
enhanced by IL-2. In a previous study, we found that hu
man recombinant IL-10 (hrIL-10) inhibited BLV tax and pol
mRNA, while IL-2 enhanced the detection of BLV tax and pol
mRNA and BLV p24 protein in PBMCs in vitro (27). Also,
IL-10-mediated regulation of BLV expression was macrophage
dependent. Because COX-2, an important enzyme for prosta
glandin synthesis, is mainly expressed by macrophages (18, 26,
36), we hypothesized that COX-2 and its products may affect
BLV expression regulated by IL-2 and IL-10. Quantitative
competitive PCR (QC-PCR) was performed to determine
COX-2 mRNA levels in PBMCs cultured with or without hrIL-
10. Transcripts of COX-2 mRNA were quantified by a com-
petitive reverse transcriptase PCR (RT-PCR) assay using stan-
dard curve methodology. Validation of this assay and synthesis
of native and competitor standards has been published previ-
ously (37, 38). A standard curve was created by RT-PCR using
a constant amount of competitor RNA (2 aM) together with
twofold serial dilutions of native RNA (15 to 0.11 aM). Un-
known mRNA samples were diluted as needed, reverse tran-
scribed and amplified with the same amount of competitor
RNA, and compared to the standard curve. Reverse transcrip-
tion was carried out in 1x RT buffer (Promega), 0.2 mM
deoxynucleoside triphosphates, 100 μM random hexamer, and
40 U of Moloney murine leukemia virus RT for 1.5 h at 37°C,
followed by 95°C for 10 min in a final volume of 20 μl. Four
microliters of RT reaction mixture were then PCR amplified
(30 s, 95°C; 30 s, 57°C; 30 s, 72°C) for 30 cycles, followed by
72°C for 5 min in 20 μl of 1x PCR buffer (Promega, Madison,
Wis.). 1.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphates,
0.4 μM upstream and downstream primers, and 0.5 U of Taq
DNA polymerase. PCR products were separated on a 5%
polyacrylamide gel electrophoresis (PAGE) gel and stained
with ethidium bromide. Bands observed in gels were quantified
using Collage® software (Fotodyne, Heartland, Wis.). Densito-
metric values for the standard curve were plotted as follows:
log10[native RNA]/[competitor RNA] versus log10 [native RNA (aM)]. The
amounts of mimic were added to the same tube and amplified
simultaneously with tubes for a standard reaction. Gel photo-
graphs were scanned, and the amplified DNA bands were
analyzed by densitometry using the NIH Image program, ver-
sion 1.61, with standard curves constructed with Cricket graph.
The amount of cytokine produced was determined by compar-
ing the density ratios of sample and standard reaction mix-
tures. As expected, we observed that different animals have
different viral loads. Usually, PL animals have more viral load
than AL animals. While IL-10 reduced detection of BLV tax
and pol mRNA, PGE2 reversed this IL-10 inhibitory effect
(Fig. 2a). Addition of PGE2 alone also enhanced BLV tax and
pol mRNA levels (Fig. 2b). These results suggest that macro-
phage-derived PGE2 may stimulate BLV expression from in-
fected B cells and that IL-10 may inhibit BLV expression by
reducing PGE2 production. At the higher concentration of
PGE2, BLV tax and pol mRNA levels were slightly diminished,
indicating that the stimulatory effect of PGE2 was maximal at

FIG. 1. IL-10 inhibits detection of COX-2 mRNA (a and b), while IL-2
stimulates detection of COX-2 mRNA (b). PBMCs from PL animals and AL
animals were cultured for 3 days with or without IL-10 and IL-2, and QC-PCR
was performed. PCR products were separated on a 5% PAGE gel and stained
with ethidium bromide. Quantification of gels was accomplished using Collage®
software. Densitometric values for the standard curve were plotted as follows:
log10[native RNA]/[competitor RNA] versus log10 [native RNA (aM)]. The
amounts of COX-2 mRNA were calculated based on the standard curve. The
data is representative of three different PL animals tested (P2 [a] and P49 [b])
and three AL animals tested (S17 [a]). Standard errors of means are shown from
at least three experiments on cells from the same animal.
To confirm the effects of PGE2 to increase BLV mRNA levels, a selective COX-2 inhibitor, NS-398 (21), was added to PBMC cultures. As expected, the level of BLV tax mRNA was dramatically suppressed by the COX-2 inhibitor (Fig. 2c). In contrast, addition of PGE2 increased BLV tax mRNA detection regardless of NS-398 addition (Fig. 2c). These data suggest that a selective COX-2 inhibitor may reduce the level of BLV mRNA in infected B cells and that PGE2 addition may bypass the NS-398 inhibition of COX-2 activity.

Purified BLV increases the level of COX-2 mRNA and has an autocrine effect that increases the levels of BLV tax and pol mRNA. Numerous factors can stimulate COX-2 and PGE2 expression by macrophages. To investigate how BLV infection might affect COX-2 and BLV expression, BLV proteins were purified from the supernatant of the BL3* cell line using metrizamide density gradient centrifugation. Purified BLV antigens were confirmed by sodium dodecyl sulfate-PAGE and immunoblotting with anti-gp51 and -p24 antibodies (data not shown). When purified BLV was added to PBMC cultures, COX-2 mRNA detection rapidly increased (Fig. 3a). Also, detection of BLV tax and pol mRNA increased by addition of purified BLV (Fig. 3b), suggesting that BLV antigens regulate BLV expression by an autocrine mechanism. To remove the possibility that other factors isolated during BLV antigen purification could affect COX-2 and BLV expression, a BLV-negative reagent was prepared from the BL3 cell line, which does not produce any BLV antigens. BLV gp51 and p24 were not detected by immunoblotting in the purified BLV-negative material. The BL3 purified material could not enhance COX-2 mRNA or BLV tax and pol mRNA (Fig. 3).

PGE2 suppresses antigen-specific PBMC proliferation. To determine if PBMC proliferation and BLV expression were correlated, different concentrations of PGE2 were added to the PBMC cultures with or without BL3* supernatant as a viral antigen source (23). Following incubation of PBMCs for 3 to 5 days, cells were harvested for further experiments. In cell prolifer...
liferation assays, [3H]thymidine was added 8 to 12 h before harvest and the radioactivity of the harvested cells was measured by a \( \beta \)-counter (MATRIX 9600; Packard, Meriden, Conn.). Medium alone was used as a control in proliferation assay. Addition of BL3* supernatant increased PBMC proliferation more than 10-fold. However, antigen-specific PBMC proliferation was dramatically suppressed with increasing concentrations of PGE2 (Fig. 4). Thus, PGE2 enhanced BLV detection but suppressed antigen-specific PBMC proliferation. The concentration of cells differed between transcription and proliferation assays, as fewer cells were optimal in the U-bottom wells for the proliferation assay.

The results presented here demonstrate that IL-10 inhibited detection of BLV \textit{tax} and \textit{pol} mRNA and reduced COX-2 transcription from macrophages, while PGE2 activated BLV \textit{tax} and \textit{pol} mRNA. These data indicate that regulation of BLV is closely related to signals induced by PGE2. Both AL and PL animals, but not noninfected animals, produced similar findings when tested with IL-2, IL-10, and PGE2, supporting a common mechanism of pathogenesis in infected animals. Also, in spite of the relatively low IL-10 levels, AL animals produced more IL-10 than uninfected animals, and AL and PL animals responded similarly to IL-10 and PGE2. These findings suggest that macrophages produce PGE2 and have a central role in regulating BLV expression in infected B cells (Fig. 5).

FIG. 3. BLV proteins stimulate COX-2 mRNA (a) and BLV \textit{tax} and \textit{pol} mRNA (b). BLV proteins were purified from BL3* supernatant, and BLV-negative reagent was prepared from BL3 supernatant using metrizamide density gradient centrifugation. BLV-infected PBMCs were cultured for 3 days with similar volumes of purified BLV (10 \( \mu \)g/ml) and BLV-negative (BLV-) materials. COX-2 and BLV \textit{tax} and \textit{pol} mRNA were quantified as described above. Shown are representative data (PL animals) of experiments with three different AL and PL animals. Standard errors of means are shown from at least three experiments on cells from the same animal.

The E series of prostaglandins are widely known as immunosuppressive products produced by macrophages, follicular dendritic cells, and fibroblasts (10, 17). These prostaglandins, especially PGE2, can downregulate many aspects of B- and T-cell functions. PGE2 production is triggered by inflammatory cytokines, such as IL-1 and TNF-\( \alpha \), that are produced in viral and bacterial infections (4, 20). Increasing PGE2 negatively regulates type 1 cytokines, such as IL-2, IFN-\( \gamma \), and TNF-\( \alpha \), by increasing production of type 2 cytokines, such as IL-10 (13). Thus, PGE2 may have a central role in regulating production of type 1 and type 2 cytokines. PGE2 activates a humoral immune response, stimulating B-cell differentiation and immunoglobulin class switching (26). In this paper, we show that PGE2 stimulated detection of BLV \textit{tax} and \textit{pol} mRNA and inhibited PBMC proliferation. These findings suggest that enhancement of BLV expression by PGE2 may not depend on cell proliferation. Ironically, B cells are the only PBMCs that are significantly infected with BLV (22), while macrophages are the only source of PGE2 in PBMCs (14). However, B cells

FIG. 4. PGE2 inhibits BLV-specific PBMC proliferation. BLV-infected PBMCs from AL and PL animals were cultured with BL3* supernatant (BLV+) or medium alone (BLV-) for 5 days with different concentrations of PGE2 (0.03 to 30 \( \mu \)M). Proliferation was assessed by [3H]thymidine incorporation using a \( \beta \)-scintillation counter. The data are representative of experiments with four different AL and PL animals. Standard errors of means are shown.
Figure 5. Possible linkage between BLV expression by B cells and COX-2 and PGE2 produced by macrophages. Arrows represent positive regulation, and a blunt line represents negative regulation.

have a number of PGE2 receptors that regulate B-cell activation (7). Signal transduction by PGE2 receptors mediates increased cyclic AMP (cAMP) production (3). BLV long terminal repeats (LTRs) contain a cAMP-response element (CRE) that facilitates BLV gene transcription (1, 39). Tax stabilizes CRE-binding protein (CREB) to bind CRE in LTRs (5). Thus, CREB and Tax may activate BLV expression of infected B cells. In addition, protein kinase C (PKC) increases BLV expression with increased Ca2+ influx (15). Therefore, increased PGE2 production by macrophages may stimulate BLV tax and pol mRNA expression through cAMP-dependent PKA and/or PKC signal transduction pathway. BLV LTRs also contain NF-kB binding sites that facilitate BLV transcription (6). Recently, antiinflammatory agents, such as aspirin and salicylate, reportedly inhibit the activity of IkB kinase-β, which facilitates the degradation of IkB and activates NF-kB (40). Antiinflammatory agents that inhibit prostaglandin synthesis may suppress BLV expression via NF-kB inhibition.

We demonstrate that BLV functions as a stimulant of COX-2 expression. Although PGE2 enhances IL-10 expression (13) to inhibit COX-2 and BLV expression, a synergistic effect of BLV expression, opportunistic infections (35), pregnancy (19), and/or stress (8) could induce disease progression in BLV infection. COX-2 and PGE2 also inhibit programmed cell death and facilitate tumor formation (33, 34), and thus these activities might promote lymphosarcoma and leukemia with other carcinogenic factors, such as Bcl-2 and BLV Tax protein, in BLV infection. Thus, in other retrovirus infections, the inhibitory function of IL-10 on human immunodeficiency virus expression has been reported (24, 30, 31). Most studies have utilized macrophage cell lines and primary macrophages, while the studies with T-cell lines or primary T cells failed to demonstrate the IL-10 inhibition of human immunodeficiency virus expression. Therefore, macrophages may have a direct role in regulating retrovirus expression responding to IL-10 (32). Our preliminary data showed that bovine herpes virus type 1 and Brucella abortus, two common opportunistic infections in cattle, activated COX-2 mRNA expression (D. Pyeon and G. A. Splitter, unpublished data). We anticipate that further research regarding PGE2 and opportunistic infections will reveal additional clues to solve the complicated mechanisms of disease progression in retrovirus infections.

To examine whether inhibitors of PGE2 or COX-2 would be efficacious for treatment of BLV infection, in vivo studies are necessary. Infected animals could be treated with a PGE2 inhibitor, such as indomethacin, and viral load and BLV expression could be measured. Treatment with a PGE2 inhibitor may reduce BLV load in BLV-infected animals and would support a role for PGE2 to stimulate BLV replication and disease progression in vivo. This study provides an additional strategy to treat retrovirus infection combined with currently available antiretroviral treatment.

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