BARD

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**Lymphocyte Response to Genetically-Engineered
Bovine Leukemia Virus Proteins in Persistently
Lymphocytotic Cattle from Israel and the U.S.**

G. Splitter, Z. Trainin, J. Brenner

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Investigators:
(Principal listed first)

Gary Splitter
Ze'ev Trainin
Jacob Brenner

Affiliated Institutions:

University of Wisconsin-Madison

Ministry of Agriculture, Veterinary
Services and Animal Health, Kimron
Veterinary Institute

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Signature
Principal Investigator

Signature
Institution's Authorizing Official

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1. **ABSTRACT.** The goal of this proposal was to identify proteins of BLV recognized by lymphocyte subpopulations and determine the contribution of these proteins to viral pathogenesis. Our hypothesis was that BLV pathogenesis is governed by the T-cell response and that the immune system likely plays an important role in controlling the outcome of infection. Our studies presented in this final report demonstrate that T cell competency declines with advancing stages of infection. Dramatic differences were observed in lymphocyte proliferation to recombinant proteins encoded by BLV *gag* (p12, p15, and p24) and *env* (gp30 and gp51) genes in different disease stages. Because retroviruses are known to mutate frequently, examination of infected cattle from both Israel and the United States will likely detect variability in the immune response. This combined research approach provides the first opportunity to selectively address the importance of T-cell proliferation to BLV proteins and cytokines produced during different stages of BLV infection. Lack of this information regarding BLV infection has hindered understanding lymphocyte regulation of BLV pathogenesis. We have developed the essential reagents necessary to determine the prominence of different lymphocyte subpopulations and cytokines produced during the different disease stages within the natural host.

We found that type 1 cytokines (IL-2 and IFN- γ) increased in PBMCs from animals in early disease, and decreased in PBMCs from animals in late disease stages of BLV infection, while IL-10, increased with disease progression. Recently, a dichotomy between IL-12 and IL-10 has emerged in regards to progression of a variety of diseases. IL-12 activates type 1 cytokine production and has an antagonistic effect on type 2 cytokines. Here, using quantitative competitive PCR, we show that peripheral blood mononuclear cells from bovine leukemia virus infected animals in the alymphocytotic disease stage express increased amount of IL-12 p40 mRNA. In contrast, IL-12 p40 mRNA expression by PL animals was significantly decreased compared to normal and alymphocytotic animals.

To examine the functions of these cytokines on BLV expression, BLV *tax* and *pol* mRNA expression and p24 protein production were quantified by competitive PCR, and by immunoblotting, respectively. IL-10 inhibited BLV *tax* and *pol* mRNA expression by BLV-infected PBMCs. In addition, we determined that macrophages secrete soluble factor(s) that activate BLV expression, and that secretion of the soluble factor(s) could be inhibited by IL-10. In contrast, IL-2 increased BLV *tax* and *pol* mRNA, and p24 protein production. These findings suggest that macrophages have a key role in regulating BLV expression, and IL-10 produced by BLV-infected animals in late disease stages may serve to control BLV expression, while IL-2 in the early stage of disease may activate BLV expression.

PGE2 is an important immune regulator produced only by macrophages, and is known to facilitate HIV replication. We hypothesized that PGE2 may regulate BLV expression. Here, we show that cyclooxygenase-2 (COX-2) mRNA expression was decreased in PBMCs treated with IL-10, while IL-2 enhanced COX-2 mRNA expression. In contrast, addition of PGE2 stimulated BLV *tax* and *pol* mRNA expression, and reversed the IL-10 inhibition of BLV expression. In addition, the specific COX-2 inhibitor, NS-398, inhibited BLV expression, while addition of PGE2 increased BLV *tax* expression regardless of NS-398 addition. These findings suggest that macrophage derived cyclooxygenase -2 products, such as PGE2, may regulate virus expression and disease progression in BLV infection, and that cytokines (IL-2 and IL-10) may regulate BLV expression through PGE2 production.

2. OBJECTIVES: The goal of this BARD grant was to characterize lymphocytes and cytokines during different stages of BLV infection. Our hypothesis is that BLV pathogenesis is regulated by the T-cell and cytokine response.

3. SPECIFIC AIMS:

1. BLV viral proteins are recognized by CD5⁺ B-cells and T-cells from cattle in Israel and the United States classified as persistently lymphocytotic.
2. A panel of bovine T-cell lines responsive to individual BLV proteins will characterize the lymphocyte phenotypes and cytokines produced.
3. Specific T-cell epitope locations within the *Tax* and *Rex* viral proteins will be mapped using Israeli and U.S. cattle with persistent lymphocytosis.

4. DESCRIPTION OF THE RESEARCH TO ATTAIN THE SPECIFIC AIMS:

Introduction

Bovine leukemia virus (BLV), closely related to human T cell leukemia virus type 1 (HTLV-1), is a type C retrovirus infecting bovine B cells and leads to enzootic bovine leukosis. Genomic structures of BLV and HTLV-1 are similarly arranged. Especially, the 3' region, that sequentially encodes the nontranslated region-*orf-tax/rex* genes, is unique to BLV and HTLV-1. In addition, the features of pathogenesis are shared by BLV and HTLV-1 as a large population of individuals become infected and develop antibodies but rarely display clinical symptoms. The disease in BLV infection is divided into three stages: 1) serologically positive, persistent lymphocytosis negative (alymphocytotic, termed AL); 2) serologically positive, persistent lymphocytosis positive (persistently lymphocytotic, termed PL); and 3) tumor bearing stages. Fewer than 5% of infected animals develop malignant lymphosarcoma, while 30% of infected animals progress to persistent lymphocytosis, where non-neoplastic B cells proliferate and leukocyte counts may exceed 10,000 cells/mm³. Most infected animals never show outward signs of disease and are referred to as asymptomatic or aleukemic. Usually a long duration is required between these disease stages, but the mechanisms of disease progression are not yet clear. This BARD project examined mechanisms that controlled or permitted progression of BLV infection.

Methodology

In the work the following materials and methods were used.

Animals.

Ten seven month old Holstein-Friesian steers tested free of common enteric pathogens, pestiviruses and BLV (Table 1).

Collection of cells.

Peripheral blood mononuclear cells (PBMC) were collected by Manufacturer's protocol No. 1077 on Histopaque 1077 (Sigma).

Hematology.

Total leukocyte and differential counts were determined by standard hematological techniques. Blood samples were collected for total and differential leukocyte counts etc., up to twenty-four weeks.

Infection of cattle with BLV.

Six animals were infected with 10⁶ bovine embryo kidney cells containing BLV (BEK-BLV), two

mock-infected with 10^6 BEK cells co-cultivated with normal PBMC (BEK-PBMC) at a passage six and two not treated. The BEK-BLV persistently infected cell line was established by co-cultivating (DMEM, 10% heat inactivated fetal calf serum, antibiotics 37°C) of primary BEK cell culture (2×10^6 cells) at passage two with PBMC (2×10^7 cells) from a local BLV seropositive cattle.

AGID test.

BLV anti-gp51 antibodies in serum were detected according to manufacturer's instructions (Leukoassay B Rhone Merieux Inc., Athens, GA, USA).

RNA isolation and RT-PCR amplification conditions.

Total RNA was isolated from uncultured PBMC cells or BEK-PBMC cells with TRI-LS reagent (Molecular Research Center, Cincinnati, OH), treated with DNase (Boehringer Mannheim), extracted with phenol-chloroform, precipitated with ethanol, and 1-5 ng were used for cDNA synthesis. RT-PCR amplification procedures were used with modifications as described (Yakobson et al., 1998). For IL-12 p40 the amplification protocol was as described (Yakobson et al., 1998). Briefly, 42 cycles, 94°C, 30 sec for denaturation, 53.5, 45 sec. for annealing, 72°C, 1 min. extension, and a final 72°C 5 min. extension cycle were used. BLV gp51 amplification was accomplished by annealing at 50°C, 30 sec. and 35 cycles of amplification. Reaction products (10 µl) were visualized on ethidium bromide stained 1.0% agarose gel.

Primer design and oligonucleotide synthesis.

Unique PCR primers for the IL-12 p40 (630 bp insert) were described (Yakobson et al., 1998). Briefly, the primers selected were targeted to human IL-12 p40 subunit, complementing bases 170 (170-189) through 800 (781-800), respectively (630-bp product), positive strand 5'-CCTGAAGAAGATGGTATCAC-3' and 5'-TCCAGGTGTCAGGGTACTCC-3' negative strand. Unique PCR primers for BLVgp51 *env* gene were described Yakobson et al., 1998 with adjacent sequences complementing bases 4859 (4859-4878) through 5761 (5742-5761), respectively (902 bp product), positive 5'-CATCATCAGATGGGTAAGTC-3' strand and 5'-GACAGGGCTAGGCCTAAGGT-3' negative strand. All primers were synthesized at GeneMed, Biotechnologies Inc., CA, USA.

Cloning, sequencing and determination of primer specificity.

PCR products of expected size observed in 1% agarose gel were sequenced. Specificity of PCR amplification was confirmed. Percentage of homology with the human counterpart IL-12 p40 for the bovine IL-12 p40 630 bp insert was 90.1%. For the BLV gp51 *env* 902 insert sequence homology was 100%.

Semi-quantitative determination of gene expression levels.

Relative levels of cytokine expression were compared with normalized actin transcripts and presented as the ratio of band intensities of the cytokine RT-PCR amplification product over the corresponding β -actin RT-PCR product (Yakobson et al., 1998).

Quantitative detection of gene expression levels by competitive RT-PCR.

Primer design and specificity of PCR products for IFN γ , IL-2, IL-10 and β -actin were as described (Covert and Splitter, 1995). Plasmids containing cytokine mimics competitor molecules for bovine IFN γ , IL-2 and IL-10 (Pyeon et al., 1996) were provided by Dr. G. Splitter to Trainin's lab. The sequences of the cytokine primers and the size of PCR amplified cytokine products and cytokine mimics are described in Table 3. The cDNA was synthesized in a total volume of 50 µl, using 5 µg of total RNA as previously described (Yakobson et al., 1998). Amplifications were performed in duplicate for each set of samples using β -actin and β -actin mimic as an internal control (data not

shown). Competitive PCR was performed in a 100 µl reaction volume containing 0.2 µM of each primer and 10 µl of cDNA (from a 100 µl cDNA stock). Three serial dilutions of the competitor cytokine mimic containing plasmid were made and 5 µl, of each were added to the PCR reaction. The reaction mixture was denatured for 5 min. at 95°C and cycled for 35 cycles at 94°C for 30 sec, 60°C for 45 sec and 72°C for 1 min. PCR products with each cytokine mimic dilution were separated on 2.5% agarose gel and stained with ethidium bromide.

Results

We investigated the role of cytokines in cellular and humoral immune responses in the outcome of Enzootic Bovine Leukosis. We have established experimental models of BLV infection in young cattle. The first and second year of the project were devoted to develop cytokine assays in newly infected young animals and to assess the kinetics and relative expression of cellular and humoral response cytokines. The third year we investigated by competitive RT-PCR kinetics and amounts of expression of genes coding for IL-12, IFN γ , IL-2 and humoral response cytokine IL-10 directing disease progression to BLV+ PL $^-$ or BLV+ PL $^+$ stages. Cytokine imbalance evaluated by short-termed cellular immune response revealed two out of six BLV infected animals developed PL $^+$ status four weeks after BLV infection, which persisted as shown by repeated testing. Initially, a strong cell-mediated immune response was induced in these animals mediated by IL-12 p40 expression one to three weeks after infection without mitogen induction of peripheral blood mononuclear cells (PBMC), while viral mRNA expression was observed two weeks following infection. Expression of IFN γ was detected beginning the first and third week after infection in BLV+ PL $^-$ and BLV+ PL $^+$ animals, respectively. However IFN γ expression decreased twenty-five fold in twelve weeks after infection in animals with BLV+ PL $^+$ and was not detected at twenty-four weeks after infection. Concomitantly, IL-10 message initially detected three weeks after infection in PBMCs of animals that developed BLV+ PL $^+$ augmented seven-fold by twelve weeks and sustained a high level of expression up to 24 weeks of observation. Animals that expressed increased and extended IFN γ mRNA one to twelve weeks after infection preserved the BLV+ PL $^+$ status up to twenty-four weeks after infection. The data suggests that early expression of IL-12 and IFN γ and continuous increased expression of IFN γ in response to BLV infection may be an important factor in resistance to leukemogenesis and is associated with long-term progression or nonprogression to PL $^+$.

Lymphocyte proliferation

Lymphocyte proliferation in animals experimentally infected and mock-infected with BLV and sentinels was observed up to twelve and twenty-four weeks after infection. All data are presented in Table 1. Animals were classified as lymphocytotic by a standard leukosis key for cattle (Mammerickx et al., 1978). Such animals remained PL $^+$ up to twenty-four weeks and one year of observation. In the group of BLV infected animals two animals (3056, 715) developed PL $^+$ status by four weeks after BLV infection which persisted. One animal (3077) developed lymphocytotic cell counts only by twenty-four weeks after infection. Two other animals (20, 22) observed only up to twelve weeks developed transient lymphocytosis between three and four weeks after infection, and were PL $^-$ up to twelve weeks after infection. One of the BLV+ PL $^-$ animals (714) observed up to twenty-four weeks sustained a non-lymphocytotic cell count. Two animals (711 and 707) mock-infected with BEK cells not containing BLV and two sentinels (3081, 3067) sustained cell counts

appropriate for their age group up to twenty-four weeks and one year of observation.
BLV gp51 transcription and amplification.

BLV mRNA expression was detected in PBMC of newly infected animals directly or after co-cultivating the cells for five days with BEK cells (Table 2). In uncultured PBMC from all infected animals BLV gp51 mRNA expression and proviral DNA amplification (Fig. 1) was continuously detectable from the second and third week after infection, respectively, and up to twelve weeks of observation. In all BLV-infected animals antibodies to gp51 antigen were detectable beginning five weeks after infection and continuously during the time of observation (not shown).

IL-12 p40 mRNA expression

We compared cytokine gene expression in PBMC of BLV infected and mock-infected animals at one week intervals during the first month, at twelve and twenty-four weeks after infection. Multiple cytokine mRNA expression was detected by RT-PCR in animals during primary BLV infection. A strong cellular immune response initiated by IL-12 p40 mRNA expression was detected in PBMC from two BLV infected animals (3056, 3077). Two animals (3056 and 3077) responded in one week after infection and two and three weeks after infection, respectively (Fig. 2, Fig. 3A). IL-12 expression was not detectable later on up to one and a half year after infection.

Expression of the genes coding for IL-1 α and TNF α were detected in all BLV-infected animals from the second week after infection. These cytokines decreased with progression of infection and were low at twenty-four weeks after infection. An increased IFN γ expression was observed in PBMC of animals 714, 20, 21. Kinetics of IFN γ expression in one animal (714) from the first week to twelve weeks after infection is shown in Fig. 3B. Kinetics and relative amounts of cytokine expression of IL-12 and IFN γ mRNAs and lymphocyte expansion in BLV and one mock-infected calf (711) is shown in Fig. 3A, B and D.

Kinetics of shift from cellular to humoral response cytokine gene expression

Concomitantly with type 1 cytokine expression, IL-10 expression in PBMC of two animals (3077, 3056) increased from three to twelve weeks after infection (Fig. 3 and 3C). Twenty-four weeks after infection, relative mRNAs expression of IL-10 (Fig. 3C) were higher than those of TNF α , IL-2 and IFN γ mRNAs. IL-10 mRNA was not detected in PBMC of the 714, 715 animals (714, 715) for twenty four weeks of observation.

Quantitative evaluation of IFN γ mRNA expression

IFN γ mRNA expression was observed in animals that preserved the PL⁻ status (714, 20, 22) from the first week after infection with BLV (Fig. 4A). These animals sustained an augmented and extended IFN γ mRNA expression. To determine the amounts of IFN γ , a competitive RT-PCR assay was used with adequate cytokine primers (Table 3). Competitive RT-PCR was performed at weekly intervals. Representative data are illustrated in Fig. 4A, at weeks one, three, four, twelve and twenty-four after infection in animals that stayed PL⁻. IFN γ expression was observed from week one, and a twenty-five-fold increase in the IFN γ mRNA expression was detectable by four weeks after infection that remained augmented up to twelve weeks after infection. At twenty-four weeks after infection, the level of IFN γ expression decreased in BLV+PL⁻ animals five-fold.

Kinetics and amounts of IFN γ mRNA expression in animals that developed PL⁺ by four (3056, 715) and by twenty-four weeks (3077) after BLV infection, respectively (Table 1) were different than from animals with PL⁻ status (714, 20, 22). IFN γ mRNA expression observed in PL⁺ animals by competitive RT-PCR was initially detected only at three weeks after infection (Fig. 4B).

In PL⁺ animals by four weeks after infection, the IFN γ mRNA expression augmented five-fold and decreased by twelve weeks after infection twenty-five-fold. By twenty-four weeks after infection, no-detectable levels of IFN γ mRNA expression were observed in PL⁺ animals.

Quantitative evaluation of IL-2 mRNA expression

IL-2 mRNA expression was observed in all experimental animals with PL⁻ or PL⁺ beginning three weeks after BLV infection and through twelve and twenty-four weeks of observation (Fig. 5, A, B). Animals that sustained PL⁻ status (714, 20, 22) augmented ten-fold IL-2 mRNA expression by twelve weeks after infection. The level of IL-2 mRNA expression decreased in BLV⁺ PL⁻ animals by twenty-four weeks after infection ten-fold and returned to the level of expression observed at three and four weeks after infection (Fig. 5A). In animals that developed BLV⁺ PL⁺ status (3056, 715, 3077), the amount of IL-2 mRNA expression augmented ten-fold by four weeks after BLV infection in comparison with BLV⁺ PL⁻ animals. However, IL-2 decreased ten- and sixty-fold by twelve and twenty-four weeks after infection, respectively (Fig. 5B).

Quantitative evaluation of IL-10 mRNA expression

IL-10 mRNA expression, concomitantly with cellular response cytokine expression started at three weeks after BLV infection in two animals (3056, 3077) that developed BLV⁺ PL⁺ status. The amount of IL-10 mRNA expression in these animals augmented seven-fold from three to twelve weeks after infection. By twenty-four weeks after infection, IL-10 mRNA expression in BLV⁺ PL⁺ animals was at the same level as at twelve weeks after infection and did not decrease (Fig. 6B). In animals that sustained BLV⁺ PL⁻ status (20, 22) IL-10 mRNA expression was initially detected later, at twelve weeks after infection (Fig. 6A). In animals 714 and 715 (Table 1) IL-10 mRNA was not detected at any disease stage. The mock-infected animals expressed low levels of cytokines for several weeks after infection (Yakobson et al., 1998).

In addition, the following results were obtained and are documented in greater detail in the appended manuscripts and publications:

1. With progression of BLV disease, the proliferation of CD4 T lymphocytes declines in response to BLV proteins. This suggests that as disease progresses, host cellular immune potential to BLV viral antigens declines allowing the virus infection to persist (**Journal of Virology, 70:7584-7593, 1996**).
2. IFN- γ , IL-2 and IL-12 are cytokines that typify the early period of BLV infection, and these cytokines are associated with the serologically positive clinical stage of disease (**Journal of Virology, 72:6917-6921, 1998**).
3. IL-10 predominates during the later clinical stage of BLV infection, and that IFN- γ , IL-2 and IL-12 decline with disease progression (**Journal of Virology, 70:5706-5710, 1996**).
4. IL-10 is the product of macrophages and not from T- or B-cells. This observation now confirms that macrophages play a role in BLV persistence. Although the BLV genome is present principally in B-cells, macrophages are now known to have an important role in this viral infection. IL-10 inhibited BLV *tax* and *pol* mRNA levels in BLV-infected PBMCs; however, the inhibitory effect of IL-10 was prevented in macrophage-depleted PBMCs confirming the importance of macrophages. These results suggest that macrophages secreted

soluble factor(s) that increase BLV mRNA levels, and that secretion of these soluble factor(s) could be inhibited by IL-10. In contrast, IL-2 increased BLV *tax* and *pol* mRNA and p24 protein production. Thus, IL-10 production by BLV-infected animals with late stage disease may serve to control BLV mRNA levels, while IL-2 may increase BLV mRNA in the early disease stage (Manuscript submitted to Journal of Virology).

5. The inhibitory effect of IL-10 on BLV expression depends on soluble factors secreted by macrophages. Thus, we hypothesized that PGE2, a cyclooxygenase-2 (COX-2) product of macrophages, may regulate BLV expression. The level of COX-2 mRNA decreased in PBMCs treated with IL-10, while IL-2 enhanced the level of COX-2 mRNA. Addition of PGE2 stimulated BLV *tax* and *pol* mRNA levels and reversed the IL-10 inhibition of BLV mRNA. These findings suggest that macrophage-derived cyclooxygenase-2 products, such as PGE2 regulates virus expression and disease progression in BLV infection. Also, a novel treatment of BLV infection could be considered using COX-2 and PGE2 inhibitors (Manuscript submitted to Proceedings of the National Academy of Sciences).
6. Established the existence of *env*-specific cytotoxic T-lymphocytes against BLV. *In vitro*-expanded peripheral blood mononuclear cell effector populations consisted mainly of $\gamma\delta$ + (>40%), CD4+ (>35%) and CD8+ (10%) T-lymphocytes. Depletion studies indicated that $\gamma\delta$ +, and not CD8+, T-cells were responsible for the cytotoxicity against autologous rVV*env*-expressing fibroblasts. Additionally, cultured effector cells lysed rVV*env*-expressing xenogeneic and rVV*env*-expressing autologous fibroblasts similarly, supporting the lack of genetically restricted killing. Restimulation of effector populations increased the proportions of $\gamma\delta$ + T-cells after culture or restimulation. These results suggest that cytotoxic $\gamma\delta$ + T-lymphocytes from non-progressed cattle recognize BLV Env without a requirement for classical MHC class I and II interactions. $\gamma\delta$ + T-lymphocytes are diverse and numerous in cattle, and here we show that they may have a surveillance role during natural BLV infection (Manuscript submitted to Journal of Virology).

Discussion

We demonstrate that cytokine production in BLV-infected animals has been polarized along with disease progression. AL animals produce IL-2, IL-12 and IFN- γ at dramatically increased levels, while PL and tumor bearing animals express IL-10 more than AL animals. We speculate in these findings that AL animals may appropriately respond to BLV infection with IL-2, IL-12 and IFN- γ , while PL and tumor bearing animals have been crippled to resolve BLV infection by certain, undetermined viral factors. Also, the imbalance of cytokine production may facilitate disease progression because failure of the host immune response against BLV may give BLV a chance to replicate without immunologic constraint. Other studies support this hypothesis, showing that antigen-specific T cell proliferation is observed in AL animals responding to BLV proteins, but disappeared in PL and tumor bearing animals. In addition, cytotoxic $\gamma\delta$ T cells isolated from PL animals could not lyse BLV Tax- and Env-presenting fibroblasts, while cytotoxic T cells from AL animals successfully remove Tax- and Env-presenting cells. These studies suggest that PL and tumor bearing animals could not properly cope with BLV infection and disease progression. However, sera from PL animals contain a higher titer of anti-BLV antibodies than those of AL animals. Therefore,

B cell response in PL animals may function properly, while cell-mediated immune response is suppressed.

However, other studies show that type 2 cytokines, especially IL-10, inhibits virus replication as well as suppresses cell-mediated immune response in HIV infection. We also demonstrate similar data that IL-10 inhibits BLV expression, while the type 1 cytokine, IL-2 activates BLV expression. Although IL-10 inhibits T cell activity to remove BLV-infected cells, IL-10 has another role to suppress disease progression of BLV infection. Thus, increased IL-10 in the late disease stage may not be the result of disease progression, but an effort of host cells to inhibit virus replication in the presence of high virus burden. Although particular proteins of retroviruses may stimulate type 2 cytokine production, we could not find any differences in IL-10 expression by PBMCs responding to whole BLV and an immunosuppressive motif of the gp30 transmembrane protein. These negative results suggest that IL-10 production by BLV-infected PBMCs may be closely regulated by host cell factors and not directly by virus proteins. Similarly, IL-10 inhibition of HIV expression is highly dependent on macrophages. Others suggested that the macrophages may functionally regulate HIV expression in response to IL-10. We show that IL-10 inhibition of BLV expression is removed when macrophages are depleted, and that high BLV expression requires the presence of macrophages. In experiment using a transwell system, BLV mRNA expression increased caused by a soluble factor secreted by macrophages. These findings suggest that macrophages have a central role in the regulation of BLV expression, as well as cytokine production. We hypothesized that one of these macrophage derived soluble factors may be PGE2, which is a critical mediator regulating cytokines and lymphocytes. PGE2 causes type 2 cytokine expression and B cell differentiation, and is produced by COX-2 in macrophages. Thus, we measured COX-2 mRNA level in response to IL-10 addition, and found that IL-10 inhibits the detection of COX-2, reversed IL-10 inhibition of BLV mRNA, suggesting that PGE2 might be one of the factors macrophages secrete for BLV expression. Similarly, PGE2 also increases HIV replication *in vivo*. B lymphocytes have PGE2 receptors that transduce signals mainly through cAMP as a mediator. Interestingly, replication of retroviruses, including BLV and HIV, can be activated by increased cAMP concentration. Therefore, we speculate that up-regulation of cAMP by PGE2 may increase BLV expression and replication by B cells. Further study regarding how PGE2 transduces the signals for BLV replication would be helpful to understand the link between BLV replication and B-cell proliferation.

DESCRIPTION OF COOPERATION:

BARD support has provided the opportunity for scientific collaboration between the laboratories in Israel and the United States on a viral disease of cattle common to both countries. Dr. Splitter visited Israel in year one of the grant to initiate collaboration and determined if he could provide reagents helpful to Israeli scientists to meet the objectives of the proposal. Oligonucleotide primer sequences for bovine cytokines as well as "mimic" plasmids for quantitative PCR, already developed by the Splitter lab, were shared with the Trainin lab. The approach to evaluating BLV infected animals was similar between the two laboratories allowing comparison of findings. T-cell proliferation assays were conducted using lymphocytes from animals from both the U.S. and Israel to evaluate lymphocyte proliferation ability during different stages of infection. Examination of cattle lymphocyte responses from both countries is important because retroviruses have high mutation rates altering potential viral epitopes recognized by T lymphocytes as has occurred for B lymphocyte BLV epitopes.

Securing animals in each stage of disease was possible because both countries have infected animals within many herds. The Israeli Lab also infected BLV calves with virus to monitor the change in lymphocyte responses within the same animals. We were interested to determine if cattle from the two countries differ in cytokine response during the defined stages of BLV infection.

Each laboratory has developed and used its own experimental techniques by shared information with the other laboratory when evaluating BLV infected animals. Using this approach, the two laboratories have confirmed that selected cytokines are associated with a particular BLV disease stage, discussed in the RESULTS section above. This has facilitated research progress that would not be possible without the unique international concept of BARD. Both laboratories feel that our present scientific achievements are due to BARD support.

MAIN ACHIEVEMENTS OF THE RESEARCH:

1. With progression of BLV disease, the proliferation of CD4 T lymphocytes declines in response to BLV proteins. This suggests that as disease progresses, host cellular immune potential to BLV viral antigens declines allowing the virus infection to persist (**Journal of Virology, 70:7584-7593, 1996**).
2. IFN- γ , IL-2 and IL-12 are cytokines that typify the early period of BLV infection (**Journal of Virology, 72:6917-6921, 1998** and Yakobson, B., Brenner, J., Ungar-Waron, H. and Trainin, Z. **Veterinary Immunology and Immunopathology. 64:207-218, 1998**), and Yakobson, B., Brenner, J., Ungar-Waron, H. and Trainin, Z. **Veterinary Immunology and Immunopathology. Submitted**).
3. IL-10 predominates during the later clinical stage of BLV infection, and that IFN- γ , IL-2 and IL-12 decline with disease progression (**Journal of Virology, 70:5706-5710, 1996**).
4. IL-10 is the product of macrophages and not from T- or B-cells. This observation now confirms that macrophages play a role in BLV persistence. (**Journal of Virology, 70:5706-5710, 1996**).
5. Addition of recombinant IL-10 inhibited BLV *tax* and *pol* mRNA levels in BLV-infected PBMCs; however, the inhibitory effect of IL-10 was prevented in macrophage-depleted PBMCs confirming the importance of macrophages. These results suggest that macrophages secrete soluble factor(s) that increase BLV mRNA levels, and that secretion of these soluble factor(s) could be inhibited by IL-10. In contrast, IL-2 increased BLV *tax* and *pol* mRNA and p24 protein production. Thus, IL-10 production by BLV-infected animals with late stage disease may serve to control BLV mRNA levels, while IL-2 may increase BLV mRNA in the early disease stage (Pyeon, D. and Splitter, G. **Manuscript submitted to Journal of Virology**).
6. We hypothesized that PGE₂, a cyclooxygenase-2 (COX-2) product of macrophages, may regulate BLV expression. In fact, the level of COX-2 mRNA decreased in PBMCs treated with IL-10, while IL-2 enhanced the level of COX-2 mRNA. Addition of PGE₂ stimulated BLV *tax* and *pol* mRNA levels and reversed the IL-10 inhibition of BLV mRNA. These

findings suggest that macrophage-derived cyclooxygenase-2 products, such as PGE2 regulates virus expression and disease progression in BLV infection (Pyeon, D. and Splitter, G. **Manuscript submitted to Journal of Immunology**).

7. Established the existence of *env*-specific cytotoxic T-lymphocytes against BLV. These results suggest that cytotoxic $\gamma\delta$ + T-lymphocytes from non-progressed cattle recognize BLV Env without a requirement for classical MHC class I and II interactions. $\gamma\delta$ + T-lymphocytes are diverse and numerous in cattle, and here we show that they may have a surveillance role during natural BLV infection (Lundberg, P., and Splitter, G. **Manuscript submitted to Journal of Virology**).
8. The availability of the recombinant BLV receptor candidate, BLVRcp1, allowed us to determine a mechanism of virus neutralization by polyclonal sera and monoclonal antibodies. Bovine sera from animals naturally infected with BLV blocked gp51 binding to recombiant BLVRcp1. In contrast, virus-neutralizing monoclonal antibodies specific for gp51 F,G, and H epitopes did not prevent gp51-receptor attachment. Furthermore, gp51 neutralization epitopes F, G, and H were accessible to antibodies following gp51 attachment to BLVRcp1. This finding implies that virus neutralization by monoclonal antibodies to defined BLV gp51 epitopes can occur subsequent to virus engagement of the receptor, while polyclonal sera can specifically block virus attachment to the receptor. In conclusion, these data suggest that cell infection by BLV is a multistep process requiring receptor binding (inhibited by polyclonal sera) followed by a second, postbinding event(s) at the cell membrane (inhibited by anti-gp51 monoclonal antibody) (**Journal of Virology, 71:3263-3267, 1997**).

PUBLICATIONS RESULTING FROM THE RESEARCH PROJECT:

1. Yakobson, B., Brenner, J., Ungar-Waron, H., and Trainin, Z. (1998). Short-termed expression of IL-12 during experimental BLV infection may direct disease progression to persistent lymphocytosis. *Veterinary Immunology and Immunopathology*. (64:207-218).
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