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Citation	Japanese Journal of Veterinary Research, 44(3), 153-163
Issue Date	1996-12-18
DOI	<a href="https://doi.org/10.14943/jjvr.44.3.153">https://doi.org/10.14943/jjvr.44.3.153</a>
Doc URL	<a href="https://hdl.handle.net/2115/2567">https://hdl.handle.net/2115/2567</a>
Type	departmental bulletin paper
File Information	KJ00002398262.pdf



## IMMUNOMODULATIVE EFFECTS OF BOVINE IMMUNODEFICIENCY-LIKE VIRUS (BIV)-INFECTION AND MIXED INFECTION OF BIV AND BOVINE LEUKEMIA VIRUS ON SHEEP

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(Accepted for publication: Oct. 18, 1996)

### ABSTRACT

Experimental bovine immunodeficiency-like virus (BIV)-infection and mixed infection of BIV and bovine leukemia virus (BLV) were performed on sheep. BIV proviral DNA and anti-BIV antibodies were persistently detected in all BIV-inoculated sheep. A slight increase in lymphocyte counts was observed in BIV-infected sheep, but the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> cells in sheep peripheral blood mononuclear cells (PBMCs) were not significantly changed. A transient decrease in lymphocyte blastogenic response to concanavalin A was observed in two of three BIV-infected sheep at 3-6 months after inoculation. From 6 months after BLV-inoculation to sheep which were previously infected with BIV, the numbers of lymphocytes expressing a tumor-associated antigen (TAA) of bovine leukosis were increased compared to those of a sheep inoculated with BLV alone. The BLV titers in PBMCs and the antibody titers against BLV from sheep infected with both BIV and BLV were higher than those of a sheep inoculated with BLV alone.

Key Words: bovine immunodeficiency-like virus, bovine leukemia virus, mixed infection, sheep

### INTRODUCTION

Bovine immunodeficiency-like virus (BIV) is a lentiviral pathogen of cattle originally isolated from a cow with persistent lymphocytosis and lymphadenopathy<sup>20)</sup>. A subsequent study has shown that BIV is genetically and antigenically similar to human immunodeficiency virus (HIV) type 1<sup>5)</sup>. Calves inoculated with BIV developed a mild lymphocytosis and a moderate lymphoproliferative reaction in the small subcutaneous lymph nodes<sup>3)</sup>. Although BIV infection can induce dysfunction of monocytes<sup>13)</sup> and

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neutrophils<sup>4)</sup> in infected cattle, BIV-inoculated calves did not develop any severe clinical symptoms<sup>3,4,13,17)</sup>. However, it is presumable that BIV-induced immune dysfunction can predispose cattle to infection with other pathogens, though little is known about the effects of mixed infections of BIV and other infectious agents on disease progression.

Previous studies have shown that sheep and goats inoculated with BIV become seropositive for BIV<sup>7,18,23)</sup>, and these animals have been considered to be susceptible for BIV. It is noteworthy that sheep is highly susceptible for bovine leukemia virus (BLV) infection<sup>9,11)</sup>, and used as an animal model for the oncogenesis of BLV and vaccine development against enzootic bovine leukosis<sup>8,9,11)</sup>. The present study was carried out to determine whether BIV-infected sheep develops any immune dysfunction and clinical symptoms, and whether BIV-induced immune dysfunction can affect the leukemogenesis of BLV.

#### MATERIALS AND METHODS

##### *BIV infection to sheep*

Age-matched five Suffolk strain sheep, four castrated male (No. 1, 3, 4, and 5) and one female (No. 2), were used in this experiment. Sheep Nos. 1 and 2 were inoculated intravenously with  $1 \times 10^8$  of bovine embryo spleen (BESP) cells persistently infected with BIV R-29 strain (BESP-BIV)<sup>6,20)</sup> at 3 months of age. Sheep No. 3 received the same BIV materials at 6 months of age. Sheep No. 5 was inoculated with  $1 \times 10^8$  of uninfected BESP cells at 6 months of age, and sheep No. 4 did not receive any materials.

To monitor BIV infection in sheep, PCR and Western blotting were performed weekly as described previously<sup>6)</sup> to detect proviral DNA in PBMCs and BIV antibody, respectively. For virus isolation from sheep inoculated with BIV, sheep PBMCs were cocultivated with uninfected BESP or Madin Darby bovine kidney (MDBK) cells for 4 to 5 days. After cultivation, expression of BIV antigens on cultivated cells was detected by Western blotting using a bovine anti-BIV serum, 1141<sup>23)</sup>.

##### *Flow cytometry analysis*

Sheep PBMCs were incubated with the mouse ascitic fluids of monoclonal antibodies (MAb) 17D (anti-sheep CD4), ST8 (anti-sheep CD8) and c143 (anti-tumor associated antigen (TAA) of epizootic bovine leukosis)<sup>1)</sup>, kindly provided from Dr. Y. Aida (Institute of Physical and Chemical Research, Tsukuba, Japan), and then the cells were stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Cappel, West Chester, PA, U.S.A.). Relative immunofluorescence of live cells were measured by a flowcytometer (EPICS Profile; Coulter, Hialeah, FL, U.S.A.) on the basis of forward and 90° light scatter profiles.

##### *Blastogenesis test*

Sheep PBMCs ( $1 \times 10^6$  cells/well) were cultivated at 37°C for 72 hr in triplicate

wells of 96-well microplates with 8  $\mu\text{g/ml}$  of concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO., U.S.A.) in RPMI 1640 medium (Gibco BRL., Gaithersburg, MD., U.S.A.) supplemented with  $5 \times 10^{-5}$  M 2-mercaptoethanol and 5% fetal bovine serum (Filtron, Brooklyn, Australia), and 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ] thymidine (ICN Biomedicals Inc., Irvine, CA., U.S.A.) was added to each well. After further cultivation for 18 hr, the cells were harvested onto fiberglass filter disks. The incorporation of [ $^3\text{H}$ ] thymidine into cells was measured with a liquid scintillation counter.

#### *Superinfection of BLV to BIV-infected sheep*

To know the effects of BIV-infection on the progress of BLV-induced disease, two sheep inoculated with BIV (Nos. 1 and 2) were reinoculated with the same BIV-materials at 31 months after the first inoculation, because the early stage of BIV-infection seems to cause hematological and immunological changes to the infected animals<sup>3,6,7,10</sup>. At five months after BIV-reinoculation, the two sheep were inoculated with  $5 \times 10^5$  of BLV-infected bovine PBMCs obtained from a cow which had been experimentally infected with BLV<sup>8</sup>. Uninfected sheep No. 4 was also inoculated with the same BLV materials at the same time.

To isolate BLV from sheep, sheep PBMCs ( $5 \times 10^5$  cells) were cocultivated with the same numbers of cc81 cells for 4–6 days under the existence of 1% of BIV-infected sheep serum for the inhibition of BIV-replication. After cultivation, numbers of syncytium formation of triplicate wells for each sample were counted by Giemsa-staining. Anti-BLV antibodies were monitored by enzyme-linked immunosorbent assay (ELISA) using BLV-antigen prepared from culture supernatant of BLV-infected fetal lamb kidney (FLK) cells<sup>21</sup> as described previously<sup>14</sup>.

#### *Antibody response against ovalbumin in sheep infected with BIV and BLV*

Two BIV and BLV-infected sheep (Nos. 1 and 2), one BLV-infected sheep (No. 4), and one uninfected control sheep (No. 5) were injected subcutaneously with 1.0 mg of ovalbumin (OVA) (Sigma Chemical Co.) with 0.25 ml of Freund's incomplete adjuvant at 5 months after BLV inoculation. Two weeks after the first immunization, the same amount of immunogen was reinjected as a booster. Serum samples were collected weekly and the antibody titers against OVA were determined by ELISA.

## RESULTS

#### *Experimental BIV infection to sheep*

By using PCR, a DNA sequence specific to BIV-*gag* region was detected in PBMCs from the three sheep inoculated with BIV at 2 weeks PI, and anti-BIV p26 antibody was detected by Western blotting at 2 weeks PI (data not shown). Though proviral DNA and anti-BIV antibodies were continually detected in sheep inoculated with BIV for more than 2 years, BIV was not reisolated from PBMCs at 3, 6 and 12 months PI (data not shown).

An increase in lymphocyte counts was observed in sheep No. 2 at 2 to 6 months

PI (Fig. 1A). The other two BIV-inoculated sheep also showed slightly higher numbers of lymphocyte counts than those of the uninfected control sheep (Fig. 1A). The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells of BIV-inoculated sheep were not significantly changed (Fig. 1B, 1C). The CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratios of whole PBMCs from BIV-inoculated sheep ranged between 1.4 to 1.7 which were not significantly different from those of uninfected control sheep.

The Con A response of BIV-infected sheep Nos. 2 and 3 was transiently decreased at 3 months PI (Fig. 2B). However, this depressed response observed in BIV-inoculated sheep recovered at 6 and 12 months PI (Fig. 2C, 2D).

#### *Experimental infection of both BIV and BLV to sheep*

Two BIV-infected (Nos. 1 and 2) and one control (No. 4) sheep were inoculated with BLV to determine the effect of BIV-infection on the progress of the BLV-induced disease. The numbers of syncytia by PBMCs from BIV- and BLV-inoculated sheep were higher than those from the sheep inoculated with BLV alone (Fig. 3A). Anti-BLV antibody titers of sheep No. 2 were higher than those of sheep No. 4 at 8 to 12 weeks after BLV-inoculation (Fig. 3B).

Six months after BLV-inoculation, increase in lymphocyte counts was observed in a sheep inoculated with both BIV and BLV (No. 2) (Fig. 4). Though morphological abnormality was not observed in the lymphocytes by Giemsa-staining, the TAA-positive PBMCs were increased in sheep inoculated with both BIV and BLV (No. 1 and 2, 24% and 65% respectively) compared to a sheep inoculated with BLV alone (No. 4, 13%) and an uninfected control sheep (No. 5, 7%).

To examine the humoral immune response against foreign proteins, two sheep inoculated with BIV and BLV, one sheep inoculated with BLV alone (No. 4), and uninfected control sheep (No. 5) were injected with OVA as an immunogen. Antibody responses to OVA of sheep Nos. 1 and 2 were not different from those of sheep Nos. 4 and 5 (data not shown).

#### DISCUSSION

Although the lentiviruses are known to be species-specific in host range, BIV seems to have a broader host range than other lentiviruses. BIV can persistently infect rabbits as well as cattle<sup>6,16,22</sup>, and can induce mild depression of immune responses in infected rabbits<sup>6</sup>. In the present study, proviral DNA and anti-BIV antibodies were continually detected in sheep inoculated with BIV for more than 2 years (data not shown), and these results strongly suggest persistent infection of BIV in sheep. Nevertheless, BIV was not re-isolated from PBMCs of the infected sheep. Presumably, though BIV can incorporate into PBMCs as a provirus, the number of BIV-positive cells are too low for virus isolation.

An increase in the numbers of CD4<sup>+</sup> cells and the ratios of CD4/CD8 in BIV-inoculated sheep at 2 months PI was reported previously<sup>7</sup>. In this study, an

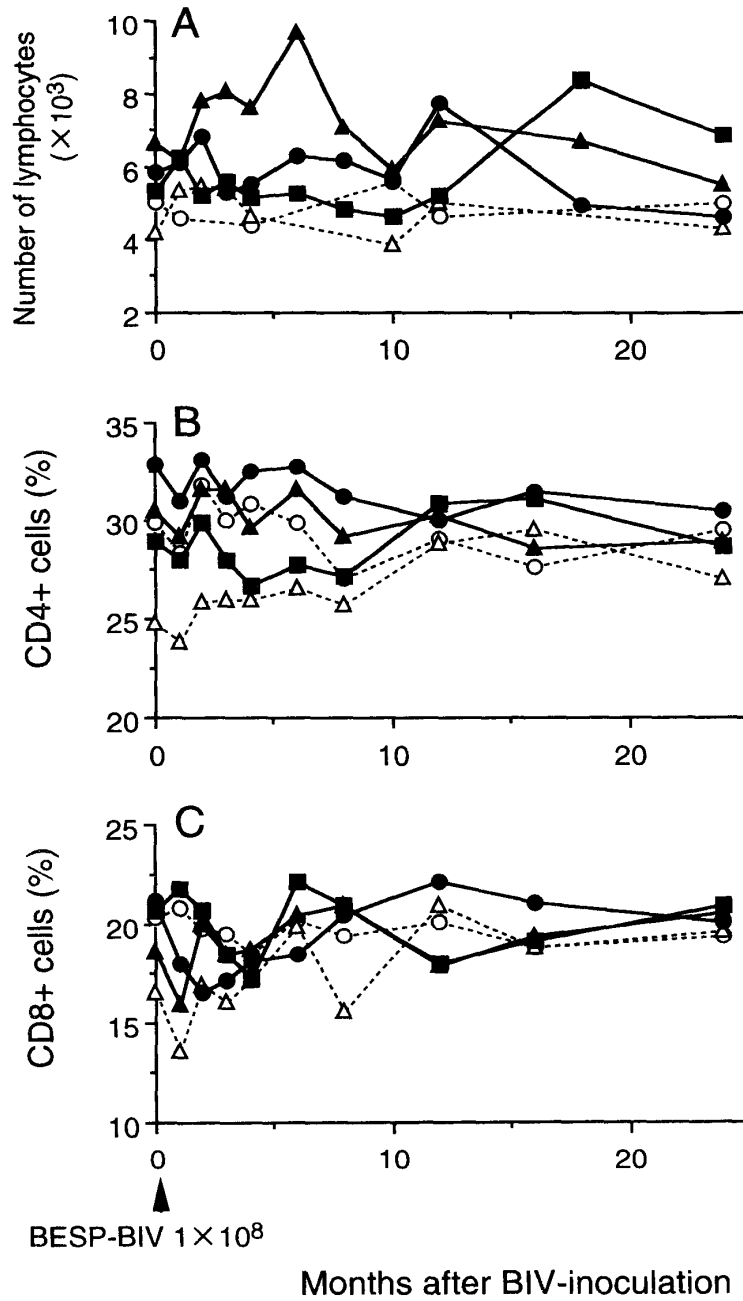


Fig. 1. (A) Total lymphocyte counts of sheep inoculated with BIV (●; No. 1, ▲; No. 2, ■; No. 3) and uninfected control (○; No. 4 and △; No. 5) sheep. (B, C) Percentages of CD4<sup>+</sup>(B) and CD8<sup>+</sup> T-cells (C) in whole PBMCs in BIV-infected and uninfected control sheep (the same symbols as A).

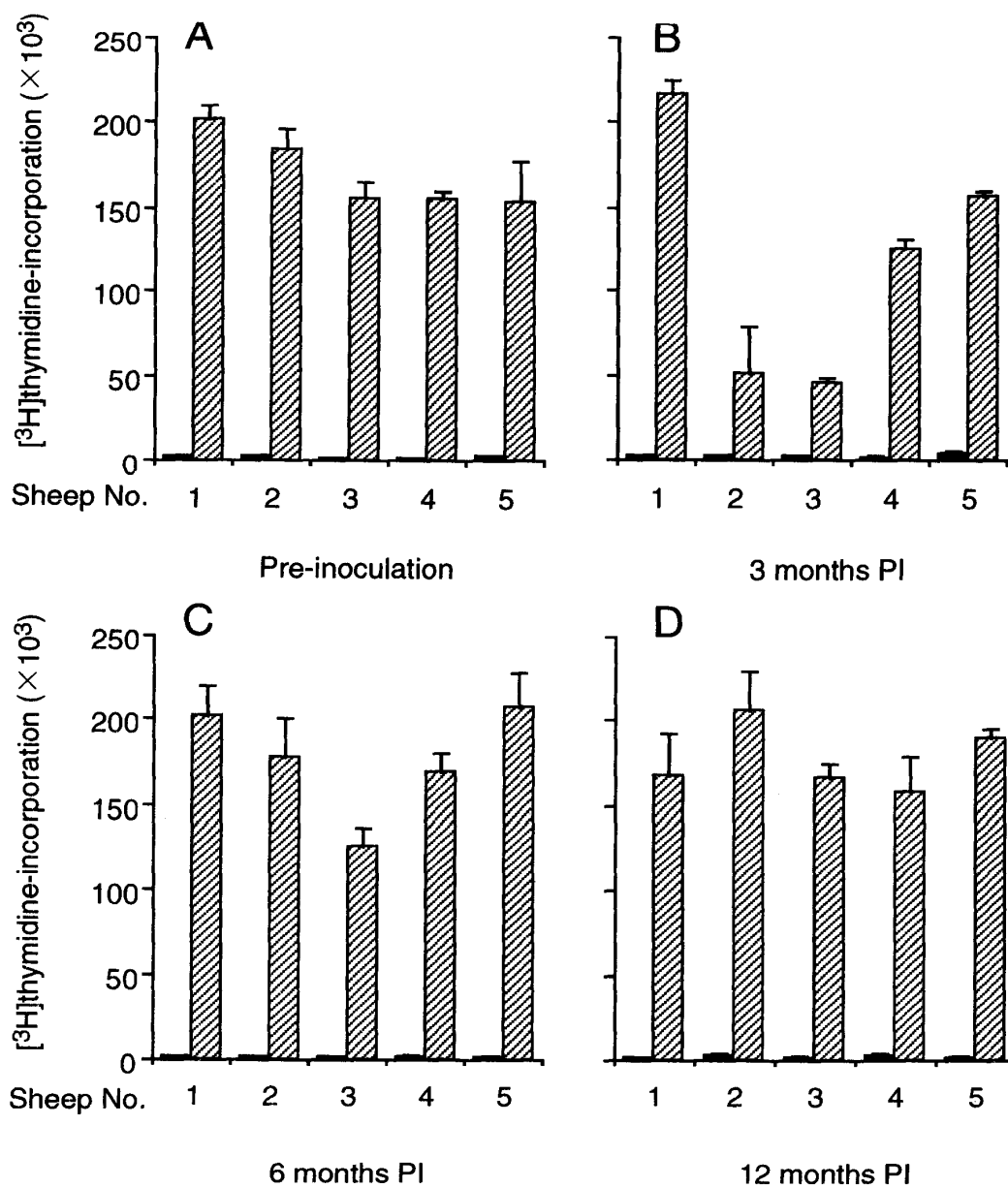


Fig. 2. Lymphocyte blastogenic response to Con A in BIV-inoculated (Nos. 1 to 3) and control (Nos. 4 and No. 5) sheep. PBMCs were cultivated with  $8 \mu\text{g/ml}$  of Con A (hatched bars) or without stimulation (filled bars) for 90 hr, and  $[^3\text{H}]$  thymidine-incorporation into cells was measured with liquid scintillation counting.

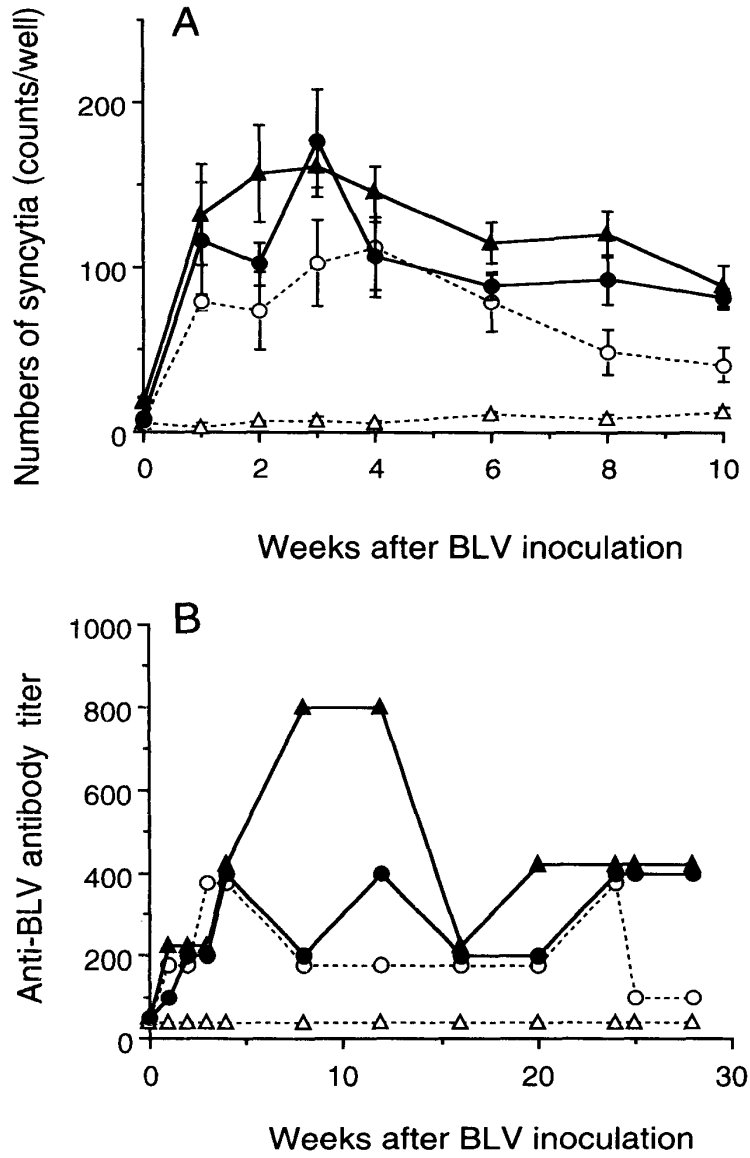


Fig. 3. (A) The numbers of syncytium formation in cultivated PBMCs from sheep inoculated with both BIV and BLV (●; No. 1, ▲; No. 2), BLV alone (○; No. 4), and uninfected control sheep (△; No. 5). Vertical bars represent standard errors. (B) Detection of antibodies against BLV in sheep inoculated with both BIV and BLV (●; No. 1, ▲; No. 2), BLV alone (○; No. 4), and uninfected control sheep (△; No. 5). Antibody titers were determined by ELISA.

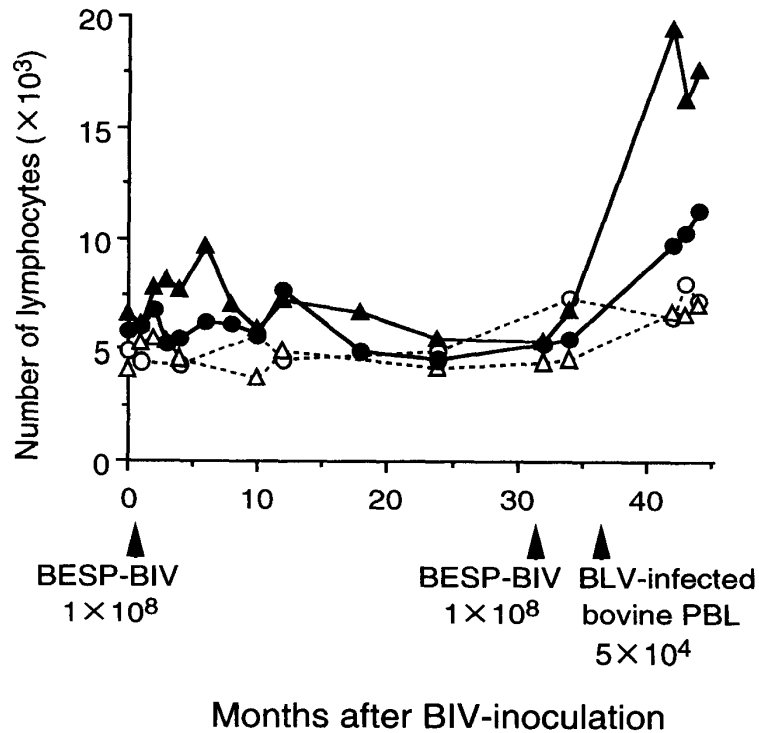


Fig. 4. Schedules of inoculation and total lymphocyte counts of sheep inoculated with BIV and BLV (●; No. 1, ▲; No. 2), BLV alone (○; No. 4), and uninfected control sheep (△; No. 5).

increase in total lymphocyte counts was observed (Fig. 1A), but the increase of  $CD4^+$  cells and the  $CD4/CD8$  ratios were not observed (Fig. 1B, 1C). The reason for this discrepancy is unclear. Differences in inoculation and other experimental designs may cause this discrepancy, or these contrasting results suggest that the hematological changes in sheep inoculated with BIV may be transient and not so drastic.

Previous studies have shown that one of the major target cells for BIV in cattle are monocytes/macrophages<sup>13,17</sup>, and some of the monocyte<sup>13</sup> and neutrophil functions<sup>4</sup> were depressed in BIV-infected calves. However, it is still unclear whether BIV infection can alter lymphocyte functions in infected animals. Suppression of lymphocyte blastogenic response to mitogens was reported in BIV-inoculated cattle at 6 months PI<sup>10</sup>, while the opposite result was also reported in BIV-inoculated calves both at 4–5 and 19–27 months PI<sup>4</sup>. In this study, depression of lymphocyte blastogenic response to Con A was observed in sheep inoculated with BIV at 3 to 6 months PI, and the depression recovered at 6 to 12 months PI (Fig. 2). These

results suggest that mitogen responsiveness in BIV-infected animals may be altered during the course of the infection.

Previously, we observed delay or decrease of humoral immune response against foreign proteins in cattle<sup>13)</sup> and rabbits<sup>6)</sup> infected with BIV. However, antibody response against a foreign protein, ovalbumin, was not altered in sheep infected with both BIV and BLV. The titers of BLV recovered from PBMCs and antibody response to BLV were increased in these sheep compared to those of the sheep inoculated with BLV alone (Fig. 4A). These results suggest that BIV-infection may support the BLV-replication. The significant increase in lymphocyte counts (Fig. 4) and TAA-positive PBMCs were observed in sheep infected with both BIV and BLV, but not in sheep infected with BLV alone. The TAA-positive cells are detected in PBMCs and lymphosarcoma cells from cattle<sup>12)</sup> and sheep (Aida et al., personal communication) infected with BLV. It is presumed that the high expression of TAA on lymphocytes is one of the important markers for BLV-induced tumors<sup>1,12)</sup>. Although the number of tested animals was limited, the present results suggest the possibility that BIV-infection may accelerate BLV-induced leukosis.

Similar enhancement of lymphoproliferative disease was reported in African green monkey infected dually with simian immunodeficiency virus and simian T-lymphotropic virus<sup>19)</sup>. Tax protein of the type I human T-cell leukemia virus (HTLV-1) can augment activity of the HIV-1 long terminal repeat (LTR)<sup>2)</sup>. Similarly, BLV can transactivate the LTR of BIV even at a very low level<sup>15)</sup>. Though the pathogenicity of BIV has been considered to be very limited, the effects of mixed infection of BIV and BLV or other infectious agents should be extensively studied.

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