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## Immune Suppression in Calves with Bovine Immunodeficiency Virus†

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**The present study was designed to evaluate the effect of bovine immunodeficiency virus (BIV) infection on immune functions and possible interactions between BIV and other bovine viruses in calves. Ten calves were inoculated intravenously with BIV, and five served as controls. An increased lymphocyte proliferation to BIV gag protein was demonstrated 2 to 6 weeks after BIV inoculation ( $P < 0.05$ ). Lymphocyte subset differentiation revealed a decreased CD4/CD8 ratio ( $P < 0.05$ ) during weeks 2 to 7, suggesting a possible immune dysfunction in BIV-infected calves. When the calves were inoculated with bovine herpesvirus type 1 (BHV-1), the antibody response to BHV-1 in BIV-infected calves was delayed and the antibody titers were significantly lower ( $P < 0.05$ ). Injection of bovine viral diarrhea virus vaccine also elicited a lower neutralizing antibody response in BIV-infected calves. The results indicated that immune suppression occurred in BIV-infected calves.**

Bovine immunodeficiency virus (BIV), a lentivirus of the family *Retroviridae*, was first isolated from a dairy cow with lymphocytosis, lymphadenopathy, neuropathy, and progressive emaciation (19). Studies have shown that BIV resembles human immunodeficiency virus and other lentiviruses, e.g., equine infectious anemia virus, in its structural, genetic, antigenic, and biological properties (1, 3, 9). Recent serological surveys have shown that BIV infection is worldwide, with the highest incidence being one of up to 60% of herd population in South America (6, 8, 11, 13, 18, 21). Although clinical disease has not been reproduced reliably by BIV experimental inoculation in cattle, BIV infections have been established successfully in cattle and other species, as shown by detectable antibody and the presence of virus in peripheral blood mononuclear cells (PBMC) (4, 7, 12, 20, 21).

The immunological effect of BIV infection in cattle has been studied by several research groups (7, 12, 14). However, the experiments were often based on a limited number of animals and the results were sometime inconsistent with each other. In one study, an increase in lymphocyte proliferation to phytohemagglutinin (PHA) was demonstrated in calves 4 months after infection (7), while in another investigation, an association of BIV infection with decreased lymphocyte proliferation to pokeweed mitogen and concanavalin A (Con A) as well as to PHA was found in calves 6 months after infection (12). A depletion of CD4<sup>+</sup> cells in two experimentally infected calves 3 to 4 months after inoculation with the original BIV strain, R-29, was reported (17). It was also noted that there was a delay in the humoral immune response against foreign proteins in three BIV-infected calves (10, 14). In this study, fifteen calves were used in the experiment in order to further evaluate the immune functions of BIV-infected calves and to investigate the effect of BIV infection on the immune responses to other

important bovine viruses, e.g., bovine herpesvirus type 1 (BHV-1) and bovine viral diarrhea virus (BVDV).

Fifteen calves 8 to 9 months old were selected for the experiment, and they were free of antibodies to bovine leukemia virus, BVDV, BHV-1, and BIV. The calves were housed in the isolation facilities at the Animal Resource in the College of Veterinary Medicine, Kansas State University. Ten of the calves were inoculated intravenously with 50 ml of BIV (isolate R-29)-infected fetal bovine lung (FBL) cells ( $10^5$  cells/ml), and five calves inoculated with 50 ml of FBL cells ( $10^5$  cells/ml) served as controls. To evaluate the effect of BIV infection on the immune responses to other bovine viruses, five BIV-infected and five non-BIV-infected calves were inoculated intranasally with BHV-1 (Cooper strain;  $2 \times 10^6$  PFU/calf) 24 weeks after BIV infection and the other five BIV-infected calves served as controls. Forty-two weeks after BIV injection, four BIV-infected and two non-BIV-infected calves were given intramuscularly a single dose of BVDV vaccine (Sanofi Animal Health Inc., Lenexa, Kans.). For immune function analysis and virus detection in calves, blood samples were collected and rectal temperature and clinical observations were recorded twice a week during weeks 0 to 8 after each treatment, i.e., BIV and BHV-1 inoculations and BVDV vaccine injection, and once a week after 8 weeks of each treatment. Nasal swabs were taken twice a week after BHV-1 inoculation for BHV-1 isolation.

For immune function assays, blood samples were collected from the jugular vein in Vacutainer tubes containing sodium heparin (Becton Dickinson, Rutherford, N.J.). Buffy coat containing PBMC was isolated by the density gradient method as described by Roth et al. (16). Mononuclear cells were finally resuspended in Dulbecco's modified Eagle medium enriched with 10% fetal bovine serum (FBS), nonessential amino acids, vitamins, and sodium pyruvate (Life Technologies, Gaithersburg, Md.) at a concentration of  $2 \times 10^6$  cells/ml. The lymphocyte proliferation assay was performed by a procedure adapted from those previously described by Clerici et al. (5). PBMC ( $2 \times 10^5$ ) were dispensed into each well of 96-well tissue culture plates (Becton Dickinson) along with four replicates of each mitogen, antigen, and control medium. In the preliminary study, the optimal final concentrations of mitogens

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and antigens were determined to be the following: 10 µg/ml for PHA (Murex Diagnostics Ltd., Hartford, England), 5 µg/ml for Con A (Sigma Chemical Co., St. Louis, Mo.), 60 µg/ml for lipopolysaccharide (Sigma Chemical Co.), 1 µg/ml for BIV recombinant *gag* protein and *Escherichia coli* fusion protein (2), and  $5 \times 10^6$  50% tissue culture infective doses (TCID<sub>50</sub>)/ml for BHV-1 and BVDV inactivated viral antigens. Undiluted BIV-FBL cell culture supernatant also was included in the assay as a control stimulator. After mitogens and antigens were added, the plates were incubated at 37°C for 72 h with 5% CO<sub>2</sub> in a humidified incubator, followed by an 18-h incubation with 1 µCi of [<sup>3</sup>H]thymidine (specific activity, 6.7 Ci/mmol; NEN, Boston, Mass.) in each well. The cells were collected by a PHD cell harvester (Cambridge Technology Inc., Cambridge, Mass.), and tritium incorporation in the cells was measured by a scintillation counter (Pharmacia/LKB, Uppsala, Sweden). The results were calculated as a stimulation index (SI), in which the mean values for disintegrations per minute (DPM) from four replicate wells of PBMC with mitogens or antigens were divided by the mean DPM values from four replicate wells of PBMC in the control medium.

In lymphocyte subset analysis, PBMC ( $10^6$  cells) suspended in each tube were washed twice with phosphate-buffered saline (PBS; 0.01 M, pH 7.2) by centrifugation at  $2,000 \times g$  for 4 min. Then, 150 µl (2 µg) of the appropriate primary monoclonal antibodies, including anti-bovine CD4, anti-bovine CD8, and anti-bovine immunoglobulin M (IgM; VMRD, Pullman, Wash.), was added to the cells in each tube. After incubation at 4°C for 30 min, the cells were washed three times with cold PBS. A second 30-min incubation at 4°C was performed in 150 µl of fluorescein isothiocyanate-conjugated goat anti-mouse IgG-IgM antibody (HyClone Laboratories, Inc., Logan, Utah) diluted 1:100. The cells were washed three more times and fixed in 0.5 ml of PBS containing 0.2% paraformaldehyde. The fixed cells were stored at 4°C in the dark until analyzed; analysis was performed within a week. Flow cytometric analysis of CD4, CD8, and B cells was performed by counting 10,000 cells from each sample on the FACscan flow cytometer (Becton Dickinson).

Detection of BIV antibody by Western blot analysis with recombinant *gag* protein as antigen was performed as described by Atkinson et al. (2). Virus isolation was performed by cocultivation of PBMC with FBL cells as previously described (21, 22). BIV infection was further confirmed by amplification of a conserved BIV *pol* gene segment from PBMC genomic DNA by PCR with a pair of designed primers (BIV 127, nucleotides [nt] 2257 to 2275, ATGCTAATGGATTTTAGGGA; and BIV 127, nt 2478 to 2497, CATCCTTGTTGGTAGAACA TT). Thermal cycling was performed in a Perkin-Elmer machine by completing 35 cycles with the following profile: 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The PCR products were analyzed on a 1% agarose gel and were confirmed by Southern hybridization.

BHV-1- and BVDV-neutralizing antibodies were detected by a virus neutralization test described by Xue et al. (23). The 50% endpoint of plaque reduction was calculated by the Reed-Munch method (15). For BHV-1 isolation, nasal swabs taken from calves were dipped into Dulbecco's medium containing 10% FBS and antibiotics. The TCID<sub>50</sub> test was performed in flat-bottom 96-well tissue culture plates as previously described (15). Briefly, the swab-washed media constituting the viral samples were serially diluted 10-fold with Dulbecco's medium containing 5% FBS, and then 100 µl of each dilution was added to each of four replicate wells of a 96-well plate; this was followed by the addition of 100 µl of Madin-Darby bovine kidney cells ( $1.5 \times 10^5$  cells/ml) to each well. The plates were

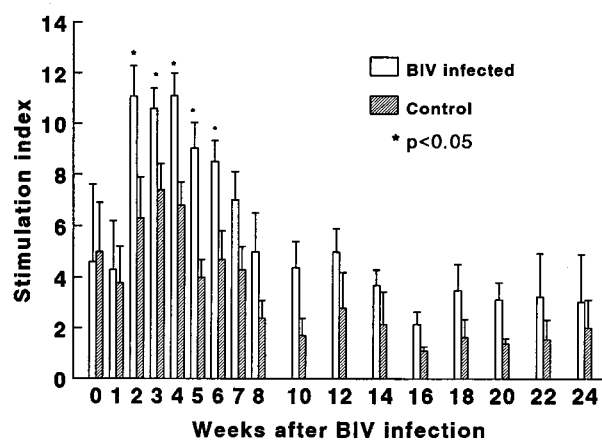


FIG. 1. Lymphocyte proliferation in BIV-infected ( $n = 10$ ) and control ( $n = 5$ ) calves stimulated by recombinant BIV *gag* protein. Data are mean SIs  $\pm$  standard errors of the means.

incubated at 37°C with 5% CO<sub>2</sub> for 3 days. Cytopathic effect was checked daily under a microscope, and TCID<sub>50</sub> was determined by the Karber Formula (15).

Experimental data was analyzed by using a *t* test for the independent samples. Differences of mean values between the treated and untreated groups in a particular time interval were evaluated. These differences were considered significant if *P* was <0.05.

Three weeks after BIV inoculation, all of the 10 BIV-inoculated calves developed antibodies specific to BIV *gag* protein, as detected by Western blot analysis, and the antibodies were detected in the calves throughout the experiment. No BIV-positive seroconversion occurred in non-BIV-infected calves. BIV could be reisolated from PBMC from the BIV-infected calves at 3 weeks postinfection (p.i.) and thereafter, but not from PBMC from control calves. PCR analysis further confirmed BIV infection in BIV-inoculated calves (data not shown). The results showed that BIV infection was established successfully in calves.

During the period of BIV infection, all calves remained clinically normal. After BHV-1 inoculation, typical symptoms of BHV-1 infection were observed in all the BHV-1-inoculated calves but not in control calves, and the symptoms disappeared within 2 weeks of BHV-1 inoculation. No abnormal clinical signs were observed after BVDV vaccine inoculation in both BIV-infected and uninfected calves. No significant hematological change was found in any of the calves during the BIV infection and other treatments.

An increased specific lymphocyte proliferation to BIV antigens, but not to mitogens and to BHV-1 and BVDV viral antigens, was demonstrated in BIV-infected calves. The SI for purified recombinant BIV *gag* protein was significantly higher for the BIV-infected calves than for control calves for 2 to 7 weeks p.i. ( $P < 0.05$ ) (Fig. 1). After 7 weeks, BIV-infected calves still tended to have a relatively high SI compared with control calves, but the difference was not statistically significant. The proliferation response to BIV-infected FBL cell culture supernatant in BIV-infected calves also tended to be higher than that in the non-BIV-infected ones during weeks 3 to 6, although this difference was not statistically significant. Lymphocyte proliferation to PHA, Con A, lipopolysaccharide, BHV-1, and BVDV antigens and to *E. coli* fusion protein was unchanged after BIV infection (data not shown).

For BIV-infected calves, a temporary but significant drop in

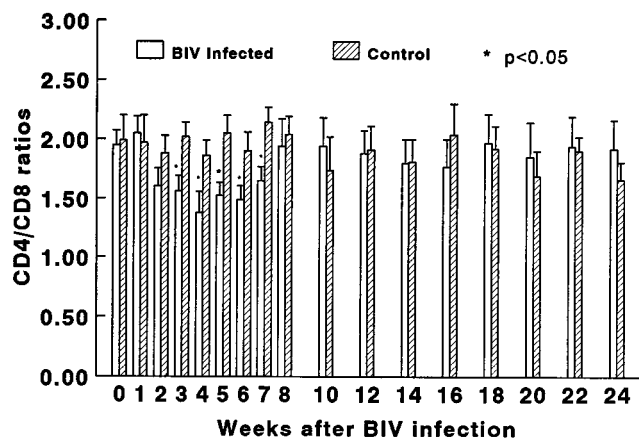


FIG. 2. CD4/CD8 ratios for BIV-infected ( $n = 10$ ) and control ( $n = 5$ ) calves. CD4<sup>+</sup> and CD8<sup>+</sup> cell numbers were determined by counting 10,000 cells by flow cytometry. Data are mean values of the CD4/CD8 ratio  $\pm$  standard errors of the means.

the CD4/CD8 ratio compared to that for control calves was observed during weeks 3 to 7 after BIV inoculation. The CD4/CD8 ratio change, shown in Fig. 2, was due to the decrease in CD4<sup>+</sup> cells and the increase in CD8<sup>+</sup> cells. For the BIV-infected calves, the mean value of the CD4/CD8 ratio dropped to 1.34 in week 4 p.i., whereas for the control calves, the mean value of the CD4/CD8 ratio was 2.0. After 8 weeks, the CD4/CD8 ratio for BIV-infected and control calves became the same.

Figure 3 shows the responses of neutralizing antibody to BHV-1 in BIV-infected and non-BIV-infected calves after BHV-1 inoculation. In the non-BIV-infected group, neutralizing antibody production started on day 7 after BHV-1 inoculation and the average antibody titer during the period from 11 to 35 days after BHV-1 infection was 1:70, while in the BIV-infected group, neutralizing antibody production started on day 11 and the average antibody titer was only 1:30. A similar result was found for the calves inoculated with BVDV vaccine. The BIV-infected calves tended to have lower neutralizing antibody titers to BVDV vaccination (Fig. 4) than the non-BIV-infected calves.

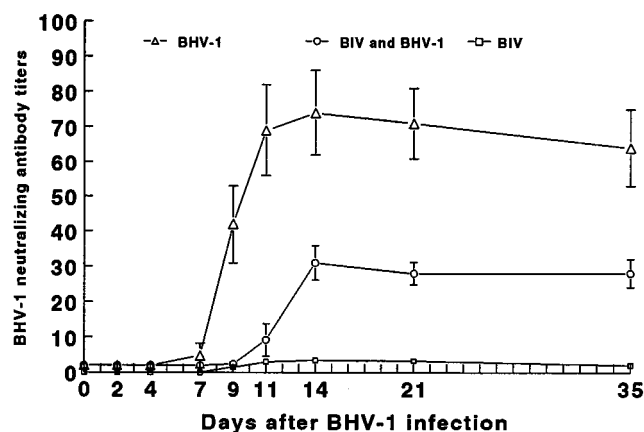


FIG. 3. Production of neutralizing antibody to BHV-1 in BIV-infected ( $\square$ ;  $n = 5$ ) and non-BIV-infected ( $\triangle$ ;  $n = 5$ ) calves after BHV-1 inoculation. BIV-infected calves without BHV-1 inoculation ( $\square$ ;  $n = 5$ ) served as controls. Data are mean antibody titers  $\pm$  standard errors of the means.

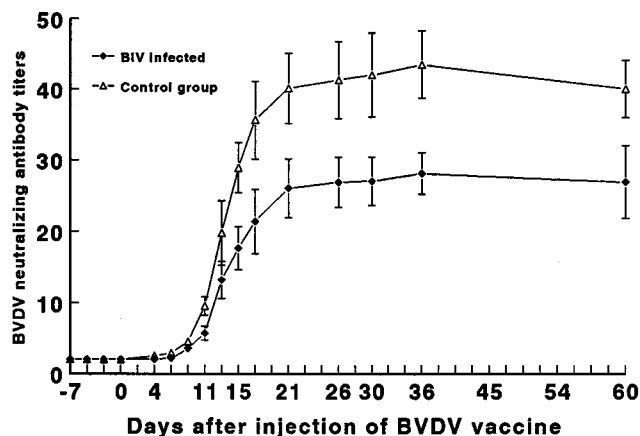


FIG. 4. Production of neutralizing antibody to BVDV in BIV-infected ( $\blacklozenge$ ;  $n = 4$ ) and non-BIV-infected ( $\triangle$ ;  $n = 2$ ) infected calves after BVDV vaccine injection. Data are mean antibody titers  $\pm$  standard errors of the means.

BHV-1 could be recovered immediately after BHV-1 intranasal inoculation, and virus titer in the nasal cavity peaked at  $10^6$  TCID<sub>50</sub>/ml within a week. The virus disappeared on day 12 after BHV-1 inoculation for both the BIV-infected and non-BIV-infected groups.

In the experiment, the SI of lymphocyte proliferation to BIV recombinant *gag* protein was significantly higher for the infected calves than for the control calves ( $P < 0.05$ ) and the SI to the BIV-FBL cell culture supernatant also tended to be higher for infected animals. However, it was also noted that the SI of recombinant *gag* protein for control calves was somewhat elevated during the first 7 weeks of BIV infection (Fig. 1). It is most likely that the calves exposed to the *E. coli* proteins in the environment before or during the BIV infection could activate the immune system. When BIV recombinant *gag* protein, containing a portion of *E. coli* fusion protein, was used as antigen, it specifically stimulated lymphocyte proliferation to both BIV *gag* and *E. coli* fusion proteins. The conclusion concerning immune system activation was supported by the observation that lymphocytes from both BIV-infected and control calves were stimulated by *E. coli* fusion protein but not by other viral proteins.

A significant decrease in CD4/CD8 ratio during weeks 3 to 7 after BIV inoculation suggests a possible immune dysfunction because of the depletion of the CD4<sup>+</sup> T-cell population by BIV infection. Although there was no direct evidence to show how BIV could act on CD4<sup>+</sup> T cells, the coincidence in the timing of the decrease in CD4<sup>+</sup> T-cell number and the rapid replication of BIV indicated a possible interaction between BIV and CD4<sup>+</sup> T cells (14). BIV-infected calves gave delayed and significantly lower neutralizing antibody responses when they were inoculated with BHV-1 6 months after BIV infection. Antibody titers to BVDV were also lower in the BIV-infected calves compared with controls after BVDV vaccine injection. Reduction and delay in the antibody responses to the two viruses observed in the BIV-infected calves may possibly be due to viral interference or immune competition among them. Other studies have also found a decrease in immune response to foreign proteins in BIV-infected calves. It was also found that this delay of immune response could be prevented by injecting serum thymic factor into BIV-infected calves (10), strongly indicating that the dysfunction in antibody response related to immune suppression occurred in BIV-infected calves.

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