Alteration of Immune Responses of Rabbits Infected with Bovine Immunodeficiency-Like Virus

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Abstract: Nine 3-month-old rabbits were inoculated with bovine immunodeficiency-like virus (BIV) to study the pathogenesis of BIV and alteration of the immune responses in experimentally infected rabbits. BIV proviral DNA and anti-BIV antibodies were detected from all rabbits inoculated with BIV-infected bovine embryo spleen (BESP) cells. Rabbits inoculated with spleen cells of the BIV-infected rabbit also converted to proviral DNA-positive and BIV-antibody-positive. The blastogenic responses to concanavalin A of peripheral blood mononuclear cells prepared from BIV-infected rabbits were not significantly different from those from uninfected controls at 2 and 4 months post-inoculation (PI). The humoral immune responses against bovine serum albumin (BSA) were depressed in two of four BIV-infected rabbits at 1 to 3 months PI. The antibody responses against sheep red blood cells (SRBCs) were significantly depressed in all BIV-infected rabbits at 2 to 4 months PI. BIV was rescued by cocultivation of spleen cells of infected rabbits with BESP cells. Distinct development of lymphoid follicle was observed in lymph nodes and spleens of uninfected rabbits which received BSA and SRBCs. In contrast, moderate lymphoid cell depletion was observed in BIV-infected rabbits which received the same immunogens.

Key words: Bovine immunodeficiency-like virus (BIV), Rabbit, Lentivirus, Immunodeficiency

A lentiviral pathogen of cattle, bovine immunodeficiency-like virus (BIV) was originally isolated from a cow with persistent lymphocytosis and lymphadenopathy (18). Subsequent studies have shown that BIV is genetically, antigenically and structurally similar to human immunodeficiency virus (HIV) type I (2, 6, 9, 20). HIV infection causes a decrease of CD4+ T lymphocytes (7, 8) and induces abnormalities in cell-mediated immune responses as well as depression of humoral immune responses (10). Some animal lentiviruses, such as simian immunodeficiency virus are also reported to cause an immunodeficiency similar to human AIDS in experimentally infected macaque monkeys (11, 23). Feline immunodeficiency virus can also induce progressive immune dysfunction in experimentally infected cats (1, 17). Calves inoculated with BIV developed a mild lymphocytosis and a moderate lymphoproliferative reaction in the small subcutaneous lymph nodes (3). Although BIV infection can induce dysfunction of monocytes and neutrophils in infected cattle (5, 14), the precise features of BIV infection is still unclear. To further characterize the pathogenesis of BIV, it is required to develop a small animal model of BIV infection. Previously, it was reported that rabbits could be infected with BIV resulting in persistent infection of BIV (15, 19), and moderate depression of T-cell region of the spleen in BIV-infected rabbits were reported (15). However there is no further precise information in experimentally BIV-infected rabbits. For these reasons, the present study was carried out to determine whether BIV-infected rabbits develop any immune dysfunction and clinical symptoms.

Abbreviations: BESP, bovine embryo spleen; BESP-BIV, BIV-infected BESP cells; BIV, bovine immunodeficiency-like virus; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FBL, fetal bovine lung; HRP, horseradish peroxidase; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PI, post-inoculation; SRBCs, sheep red blood cells.
Materials and Methods

Virus and cell cultures. Fetal bovine lung cells infected with the R29 isolate of BIV (FBL-BIV) were kindly provided from Dr. M.J. Van Der Maaten (National Animal Disease Center, Department of Agriculture, Ames, Iowa, U.S.A.). BIV-R29 was propagated by cocultivation of FBL-BIV cells with uninfected bovine embryo spleen (BESP) cells as described previously (13). BESP cells infected with BIV (BESP-BIV) were used for the inoculation of rabbits.

Experimental animals and virus inocula. Three-month-old male Japanese albino rabbits (CSK strain) were used in this experiment. Inocula of these rabbits are shown in Table 1. Seven rabbits (Nos. 1 to 7) were inoculated with BESP-BIV cells (1×10^7) intravenously and two rabbits (Nos. 8 and 9) were inoculated with spleen cells (1×10^8) from a BIV-infected rabbit (No.2) collected at 18 weeks post-inoculation (PI). Two rabbits (Nos. 11 and 12) received uninfected BESP cells (1×10^7) and two non-treated rabbits (Nos. 10 and 13) were used as controls. After inoculation, peripheral blood mononuclear cells (PBMCs) and serum samples were collected from these rabbits weekly. Hematological tests were performed every two weeks for 2 months PI, once per month after 2 months PI. All rabbits were kept under conventional conditions.

Detection of BIV proviral DNA. To monitor the infection of rabbits with BIV sequentially, we used the polymerase chain reaction (PCR) to detect the BIV proviral DNA from PBMCs and Western blotting to detect the antibody responses against BIV proteins. At the time of necropsy, detections of BIV and BIV proviral DNA from tissues of BIV-infected rabbits were attempted.

To detect proviral DNA, blood samples in citrate-phosphate-dextran as anticoagulant were obtained from BIV-inoculated and control rabbits weekly. PBMCs were isolated by Ficoll-Conray density gradient centrifugation. Collected PBMCs were resuspended in 1 ml of SET buffer (150 mM NaCl, 50 mM Tris, 10 mM EDTA+2Na, pH 8.0), and then 100 μl of 5 M NaClO4 and 33.5 μl of 10% SDS were added to lyse the cells. Then, the DNA was extracted with phenol and chloroform-isoamylalcohol (24:1) and dialyzed against 0.1× SSC (15 mM NaCl and 1.5 mM sodium citrate) for overnight at 4°C. The first PCR reaction was performed using the primer pairs specific for the BIV gag region (nt 802→821 [5’-GACCTCAGAAGCAG-CTTAA-3’] and 1198→1217 [5’-AAGTCTGTGTACGGCTTCTC-3’]) with GeneAmp PCR reagent kit (Perkin-Elmer Cetus, Norwalk, Conn., U.S.A.). Then the PCR products were amplified with another primer pair specific to the inner region (nt 854→873 [5’-TTGACCTTGCAGTGAAT-3’] and 1108→1127 [5’-AGCACCCAGTCTGGAAGT-3’]) of the sequence amplified by the former primer pairs. The amplified products were visualized by ethidium bromide staining on a 1% agarose gel, transferred to a nylon membrane (Hybond-N; Amersham Co., Buckinghamshire, U.K.) and probed with 32P-labeled inner fragments.

At the time of necropsy, spleen, lymph nodes, kidney and liver were collected for the detection of BIV by PCR. Peritoneal and alveolar cells of two rabbits (Nos. 2 and 8) were also collected by washing with warmed PBS. Adherent cells were obtained by the incubation in plastic culture dishes at 37°C for 1 hr. These adherent cells (macrophage fraction) were also tested by PCR.

Detection of BIV antibody. Serum samples were collected weekly and BIV antibody was monitored by Western blotting. Antigen for the Western blotting was prepared from supernatant fluids of BIV-infected BESP cells and MDBK cells as described previously (21). The antigen was separated on 13% sodium dodecyl sulfate-polyacrylamide gels and transferred electrophoretically to 0.45-μm-pore size polyvinylidene difluoride filters (Immobilon; Millipore, Bedford, Mass., U.S.A.). The blots were blocked with 3% gelatin in PBS. After washing, the blots were incubated with 1:100 dilution of rabbit sera for 1 hr at room temperature. The bound antibody was detected with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (ICN Immunobiologicals Inc., Lisle, Ill., U.S.A.).

Blastogenesis test. PBMCs of 4 BIV-infected rabbits (Nos. 4 to 7) and 4 control rabbits (Nos. 10 to 13) were collected at two and four months PI. The mitogenic responses were determined using 100 μl of cells (1×10^6 cells/well) in triplicate wells of 96-well microplates with 0.8 μg of concanavalin A (Con A) (Sigma Chemical Co., St. Louis, Mo., U.S.A.). The cells were incubated at 37°C for 72 hr and 1 μCi of [3H]thymidine (ICN Biomedicals Inc., Irvine, Calif., 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 [3H]thymidine into cells was measured with a liquid scintillation counter (Beckmann LS6000SC;
Humoral immune response against foreign proteins in BIV-infected rabbits. To investigate the humoral immune response against foreign proteins in BIV-infected rabbits, antibody titers to bovine serum albumin (BSA) and sheep red blood cells (SRBCs) of BIV-infected rabbits were compared with those of uninfected rabbits. Four BIV-infected rabbits (Nos. 4, 5, 6 and 7) and four control rabbits (Nos. 10, 11, 12 and 13) were injected intravenously with 1.0 mg of BSA (Sigma Chemical Co.) at 25 days after BIV inoculation. At 60 days after BIV inoculation, 1×10^8 cells of SRBCs were injected intravenously. Two weeks after the first immunization, the same dose of each immunogen was reinjected as booster. Serum samples were collected weekly and the antibody titers against BSA and SRBC were determined by enzyme-linked immunosorbent assay (ELISA) using a 1:1,000 dilution of HRP-conjugated anti-rabbit IgG. To prepare the SRBC membrane antigen for the ELISA, 10 ml of sheep erythrocytes were suspended in 200 ml of 10 mM NaH_2PO_4 (pH 7.4) and incubated for 5 min on ice. Then 20 ml of 1 M glucose was added and stirred further 2 min. The suspension was centrifuged 10,000×g for 10 min at 4°C. The precipitates were collected, and the same procedures were repeated three times. The resultant precipitates were dissolved in PBS, sonicated and used as the SRBC membrane antigen. The endpoint of ELISA was estimated from the dose-response curve and the antibody titer was expressed as the reciprocal of the maximum dilution of antibody giving A_{405}>0.2.

Virus isolation. For virus isolation, 1×10^6 cells from spleen, lung, kidney and PBMCs of four BIV-infected rabbits (Nos. 4, 5, 7 and 9) and one control rabbit (No. 13) were cocultured with 1×10^6 cells of uninfected BESP cells for 4-5 days. After cultivation the cells were collected and BIV antigen was detected by Western blotting using the bovine anti-BIV serum 1141 (22), kindly provided from Dr. M.J. Van Der Maaten.

Statistical analysis. The statistical significance of data was evaluated by Student’s t-test.

Results

Detection of Proviral DNA and BIV Antibody

By using the PCR, an expecting 300-base-pair nucleotides were detected in DNA samples of PBMC from six rabbits inoculated with BESP-BIV at 14 days PI, and the same size product was detected from the remaining rabbit at 21 days PI. These amplified products were identified as the BIV gag-specific sequences by Southern blotting and the products were continually detected (Fig. 1, Table 1). BIV-specific sequences were also detected from two (Nos. 8 and 9) rabbits which were injected with the spleen cells of BIV-infected rabbit (lanes f-k; collected at 7,14,21,28,35 and 42 days PI), but not from the mock-inoculated (lanes m and n) and non-treated (lanes l and o) rabbits. Molecular mass standards are shown on the left.

These amplified products were identified as the BIV gag-specific sequences by Southern blotting and the products were continually detected (Fig. 1, Table 1). BIV-specific sequences were also detected from two (Nos. 8 and 9) rabbits which were injected with the spleen cells of a BIV-infected rabbit (No. 2).

BIV-infected rabbits converted to seropositive at 2 to 3 weeks PI, the same time or 1 week after the rabbits turned to provirus-positive (Table 1). The major detectable antibody was antibody to BIV p26, and anti-p18 antibody appeared later (Fig. 2). The antibody was continually detected throughout the observation periods.
Hematological and Clinical Findings in BIV-Infected Rabbits

The numbers of red blood cells, lymphocytes and monocytes were sequentially monitored throughout the observation period. There were no significant differences between control and infected groups. General depilation was observed in two rabbits (Nos. 6 and 8, Table 1) inoculated with BIV from 3 months PI. Emaciation was also observed in a rabbit (No. 6, Table 1). This rabbit died at 131 days PI but no distinct changes were observed in the parenchymal tissues and the cause of death could not be determined (Table 1).

Blastogenic Responses in Infected Rabbits

The Con A responses of PBMCs in BIV-infected rabbits were tested at 2 and 4 months PI. Blastogenic responses of 4 BIV-infected (Nos. 4, 5, 6 and 7) and 4 control (Nos. 10, 11, 12 and 13) rabbits were compared. [3H] thymidine incorporation of BIV-infected rabbits was not significantly different from those of control rabbits at 2 and 4 months PI (Fig. 3).

Humoral Immune Responses against Foreign Proteins

To examine the immune responses in BIV-infected rabbits, BIV-infected rabbits and control rabbits were injected with both BSA and SRBC as immunogens. Antibody responses to BSA of two BIV-inoculated rabbits (Nos. 5 and 6) were lower than those of control rabbits at 3 to 8 weeks after first immunization, while the titers of the other two BIV-infected rabbits (Nos. 4 and 7) were the same level or higher than those of control rabbits (Fig. 4a). On the other hand, antibody responses to SRBC were significantly suppressed in all BIV-infected rabbits (Fig. 4b).

Detection of BIV from the Tissues of BIV-Infected Rabbits

At necropsy, tissues were collected from 4 BIV-infected rabbits (Nos. 4, 5, 7 and 9) and one control rabbit (No. 13), and used for virus isolation. Collected cells from these tissues were cocultured with uninfected BESP cells and expression of BIV antigen was assessed by Western blotting. Though the proviral DNA was detected in the PBMCs and some tissues of the BIV-infected rabbits by PCR (Table 2), BIV was only isolated from spleen cells.
of two rabbits (Nos. 4 and 7, Fig. 5) but not from other tissues (Table 2). Provirus DNA was also detected in adherent (macrophage) fractions of alveolar and peritoneal cells (Table 2).

**Histological Findings**

The rabbits, which were used in the comparison of the humoral immune responses against BSA and SRBC, were examined histologically. Distinct development of lymphoid follicle was observed in
lymph nodes (Fig. 6b) and spleens (Fig. 6d) of the control rabbits which received BSA and SRBCs (Table 3). However, no distinct lymphoid follicle was observed in BIV-infected rabbits which received the same immunogen, and marked follicular and paracortical lymphoid cell depletion was observed in the lymph nodes of BIV-infected rabbits (Fig. 6a, Table 3). There were no specific changes in other tissues.

Discussion

Although cattle is the natural host of BIV, BIV can infect other animals such as rabbits, sheep and goats (15, 19, 22), but the information about the pathogenesis and immune dysfunction of BIV-infected animals is limited. In the present study, we observed mild depressions of immune responses in the BIV-infected rabbits. BIV was isolated from spleen but not from lung, kidney and PBMCs of the BIV-infected rabbits (Fig. 5, Table 2). Previously, BIV was isolated from lymph nodes and PBMCs as well as spleens of the infected rabbits (15). In the present experiment, though the proviral DNA was consistently detected in PBMCs and inconsistently in lymph nodes and other tissues (Table 2), BIV was only isolated from the spleen. Although the reason why we could not rescue BIV from provirus-positive tissues as well as PBMCs is not clear, it may be due to the limited number of BIV-positive cells in

Fig. 6. Histological changes in lymph nodes and spleens of BIV-infected and uninfected rabbits. (a) Lymph node of BIV-infected rabbit No. 6. Marked diffuse lymphocyte depletion. Note that the cortex and paracortical area of the lymph node can not easily be differentiated. HE stain ×100. (b) Lymph node of control rabbit No. 12. Lymphoid follicles (arrowhead) with germinal center. HE ×100. (c) Spleen of BIV-inoculated rabbit No. 4. There is a prominent decrease in the number of lymphocytes in the white pulp. HE ×150. (d) Spleen of control rabbit No. 12. The white pulp is filled with variously sized lymphocytes and reticular cells.
these tissues compared to the spleen. Two rabbits (Nos. 8 and 9) received spleen cells of BIV-infected rabbit converted to BIV provirus-positive and antibody-positive (Figs. 1, 2, Table 1). The successful passage of BIV through rabbits was also reported by other workers (15), and the results would indicate the usefulness of the rabbit-BIV system as a small animal model of BIV infection.

Antibody responses to SRBC were significantly depressed in all 4 BIV-infected rabbits as compared with those of control rabbits (Fig. 4b). On the other hand, antibody responses to BSA of 2 BIV-inoculated rabbits were lower than those of control rabbits, however those of the other 2 BIV-positive rabbits were the same level or higher than those of control rabbits (Fig. 4a). This may reflect individual differences in the immune response. However, the antibody titers against BSA of BIV-infected rabbits were slightly higher than the average titer of control rabbits at 1 week after first BSA inoculation (Fig. 4a). Four BIV-infected rabbits (Nos. 4, 5, 6 and 7) and two control rabbits (Nos. 11 and 12) were injected with BESP cells cultured with fetal bovine serum. Thus the possibility that the rabbits may not be completely naive to BSA at the first immunization is not ruled out, and this may affect the antibody responses to BSA. The immunization times of BSA and SRBCs after BIV inoculation may also affect the results; BSA was first injected at 4 weeks PI while SRBCs were first injected at 9 weeks PI.

The depression of humoral immune responses in BIV-infected rabbits was coincident with the histological findings. Distinct development of lymphoid follicles was observed in the lymph nodes and spleens of the control rabbits which received BSA and SRBCs (Fig. 6b, 6d, Table 3), indicating that the lymphoid tissues were reacted to foreign antigen stimulation. However, no distinct lymphoid follicle was observed in BIV-infected rabbits which received the same immunogen, and lymphoid cell depletion was observed in lymphatic follicles and paracortex of BIV-infected rabbits (Fig. 6a, Table 3). These histological findings support that the immune responses against foreign antigens were depressed in BIV-infected rabbits.

The mitogenic responses of PBMCs in BIV-infected rabbits were not significantly different from those of control rabbits at 2 and 4 months after inoculation (Fig. 3). In the case of experimental BIV infection to calves, a decrease in the lymphocyte blastogenic responses to mitogens at 6 months after BIV inoculation was reported previously (12) while the opposite result was also reported in calves both at 4–5 and 19–27 months after BIV inoculation (5). Thus, it is still unclear whether BIV infection could alter the lymphocyte blastogenic responses in infected animals.

We previously showed that the major target cells of BIV in experimentally infected calves are monocytes/macrophages, and some of the monocyte functions such as superoxide anion release, phagocytic activity, and chemotactic responsiveness were depressed in BIV-infected calves (14). Neutrophil dysfunction, such as antibody-dependent-cell-mediated cytotoxicity and neutrophil iodination of BIV-infected cattle has also been reported (5). In BIV-infected rabbits, BIV-antigen-positive cells in the spleen appeared to be macrophages (15). In the present experiment, we also detected proviral DNA in adherent (macrophage) fractions of alveolar and peritoneal cells. Thus, one of the target cells of BIV in rabbits are macrophages/monocytes and the infection of BIV in macrophages may cause immune dysfunction, as suggested in BIV-infected calves (14).

A new wild isolate of BIV was reported recently (16), and the calves inoculated with this new isolate developed a transient increase of mononuclear cells but did not develop severe clinical symptoms, similar to the calves which received the BIV R29 strain. In the field, the outbreak of “bovine AIDS” in the BIV-seropositive cows in Britain was reported recently (4). These cows showed a wide range of clinical spectrum including nerve degeneration, weight loss, mouth ulcers, and respiratory infections. Concurrent infections and other physical conditions of the host may affect the pathogenesis of BIV. More information is necessary for further characterization of the pathogenesis and immunological effects of BIV in cattle. The rabbit-BIV system would be a suitable small animal model of BIV infection, and this system may also contribute to study the persistent infection of lentiviruses and lentivirus-induced immunodysfunctions.

References


