Characterization of Early Pathogenic Effects after Experimental Infection of Calves with Bovine Immunodeficiency-Like Virus

SUSAN CARPENTER,1*, LYLE D. MILLER,2 SOREN ALEXANDERSEN,1† CECELIA A. WHETSTONE,1 MARTIN J. VANDERMAATEN,3 BIRGITTE VIUFF,4 YVONNE WANNEMUEHLER,1 JANICE M. MILLER,5 AND JAMES A. ROTH3

Department of Microbiology, Immunology and Preventive Medicine1 and Department of Veterinary Pathology,2 College of Veterinary Medicine, Iowa State University, Ames, Iowa 50011, and Agricultural Research Service, U.S. Department of Agriculture, National Animal Disease Center, Ames, Iowa 500105

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Lentiviruses are exogenous, nononcogenic retroviruses which induce chronic, persistent disease in infected individuals. The lentivirus family includes the visna-maedi virus, caprine arthritis-encephalitis virus, equine infectious anemia virus, bovine immunodeficiency-like virus (BIV), and the human (HIV), simian (SIV), and feline (FIV) immunodeficiency viruses. Clinical manifestations of lentivirus infections vary widely, both among the different members of the lentivirus family as well as among individuals inoculated with the same virus. In general, lentivirus disease has been described as primary and secondary (27). Primary disease is found in all lentivirus infections and is associated with increased viral replication in macrophage populations in affected tissues. The differences in clinical disease among the various members of the lentivirus family are due primarily to the target organ affected and, in some cases, to the tempo of virus replication and induction of disease. Secondary disease is associated with viral replication in CD4+ lymphocytes and immune suppression (27). Thus far, secondary disease has been described only for primate and feline lentivirus infections.

BIV is a member of the lentivirus family which has received only limited in vivo study (38, 39). In 1972, VanDerMaaten et al. reported the isolation of a virus from cattle with persistent lymphocytosis (38). At necropsy, affected cattle were found to exhibit lymphoid hyperplasia and mild lymphocytic perivascular cuffing in the brain. Experimental infection of colostrum-deprived calves resulted in mild transient leukocytosis and moderate lymphadenopathy. Electron microscopic examination demonstrated that the causative virus was morphologically similar to visna virus (11, 38). More recent studies have shown that the bovine visna-like virus is antigenically and genetically related to other members of the lentivirus subfamily of retroviruses, including HIV, and in 1987 the virus was named bovine immunodeficiency-like virus (21). The structural similarities between BIV and HIV, as well as the occurrence of lymphadenopathy in experimentally infected calves, suggests that BIV may provide a useful animal model for HIV pathogenesis. However, few studies of BIV pathogenesis in experimentally infected calves have been reported since the original description of VanDerMaaten et al. (38). Those initial studies of experimental BIV infection identified a transient leukocytosis within the first 1 to 2 months following infection. In addition, BIV could be reisolated from experimentally infected calves during the first 2 months postinfection; however, samples collected at 16 weeks postinoculation (p.i.) or later were consistently negative. These data suggested that an early acute phase, similar to that observed in infections with several other lentiviruses, including equine infectious anemia virus (22), HIV (18), SIV (25), and FIV (44), may be characteristic of BIV infections in vivo. Therefore, we undertook this study to examine the in vivo effects of BIV during early periods following experimental infection of calves.

MATERIALS AND METHODS

Virus and cells. All BIV inocula used in these studies were derived from the original R29 isolate of BIV, the only reported field isolate of BIV. Stocks of BIV R29 had been passaged more than 25 times in primary cultures of bovine embryonic spleen (BESP) and fetal bovine lung (FBL) cells prior to use in these studies. The molecularly cloned BIV 106 isolate, derived from BIV R29 (12), was obtained as a 50-ml stock of cell-free virus from Matthew Gonda, NCI-Program Resources, Inc., Frederick, Md., and was used in these studies without further in vitro passage. The FBL-BIV R29 inoculum was prepared by cocultivation of BIV R29-infected FBL cells with noninfected FBL cells as previously described (42). This method of in vitro propagation of BIV was
previously shown to result in a significant increase in BIV antigen production (42). All of the BIV inocula were known to contain noncytopathic bovine viral diarrhea virus (BVDV), a common contaminant of commercial lots of fetal calf serum used in animal cell culture (10).

Madin-Darby bovine kidney cells (MDBK; ATCC CCL-22) were grown in Dulbecco modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% newborn calf serum, and antibiotics. Primary BESP and FBL cultures were established and maintained as previously described (24, 42). MDBK and FBL cultures were repeatedly tested and shown to be free of BVDV.

**Animals and inoculations.** Holstein calves, 2 to 4 months of age, were purchased from a local dairy and used for in vivo studies. All calves were serologically determined to be free of BVDV and bovine leukemia virus (BLV) prior to experimental inoculation. In the first series of experiments, BIV R29 propagated in BESP cells was serially passed through five calves. The passage 1 (p1) calf was inoculated intravenously (i.v.) with approximately 10⁷ BIV-infected BESP cells in 40 ml of culture supernatant. Two weeks p.i., 100 ml of whole blood was collected from this calf and immediately inoculated i.v. into a second calf. Previous studies indicated that BIV was consistently reisolated from experimentally infected calves at 2 weeks p.i. (38). Subsequent passages of 100 ml of whole blood into recipient calves were done at 2 to 3 weeks p.i. In the second experiment, the molecularly cloned BIV 106 isolate was serially passed through three calves. The p1 calf was inoculated i.v. with 40 ml of cell-free supernatant containing BIV 106. As before, 100 ml of whole blood was passed 2 weeks p.i.

A third experiment was designed to address the effect of BVDV present in the BIV inocula. All calves in this experiment were vaccinated with killed BVDV vaccine (EXCEL 4; Bio-Ceutic, St. Joseph, Mo.) at 2 and 4 weeks preinoculation. Four colostrum-deprived Holstein calves, 4 to 6 months of age, were inoculated with 1.8 × 10⁸ syncytium-forming units of BIV R29 propagated in FBL cells (FBL-BIV R29). Three colostrum-deprived age-matched calves were inoculated with FBL cells as negative controls. To control for the effects of BVDV present in the BIV inocula, the noncytopathic Nebraska isolate of BVDV (32) was included in the FBL control inocula.

**Clinical and pathological signs of BIV infection.** Blood samples were collected and rectal temperatures were recorded at least three times per week. Hematological analyses included total and differential leukocyte counts. The normal range of lymphocyte and neutrophil counts was determined from a total of 123 samplings from age-matched calves. For the purposes of this study, the normal range for the lymphocyte and neutrophil counts was defined as the range that contained 95% of the values from these control animals.

Animals in the BIV R29 serial passage experiments were euthanized at 5 to 6 weeks p.i. and examined for gross and histopathological lesions. Also included was a single control calf inoculated with 2.5 × 10⁷ peripheral blood mononuclear cells (PBMC) from an allogenic calf free of BIV, BLV, and BVDV. At necropsy, representative specimens obtained from the lymphoid system, thoracic and abdominal viscera, central nervous system, and femoral bone marrow were fixed by immersion in 10% neutral buffered formalin. Fixed tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Lymph nodes were examined for structural alterations in follicular, paracortical, medullary cord, and sinus compartments (6, 30, 40). Hemal nodes and other lymphoid tissues were examined for relative content of follicular and nonfollicular lymphoid elements (26, 28).

**Virus isolation.** For virus isolation, whole blood was collected into acid citrate dextrose at 2 and 6 weeks p.i., and PBMC were isolated by centrifugation, collection of theuffy coat, and flash lysis of the erythrocytes in the buffy coat as previously described (33). This resulted in a population of greater than 90% mononuclear cells. A total of 10⁵ to 10⁶ PBMC were cocultivated with 5 × 10⁶ BESP or FBL cells, and BIV replication was detected by syncytium formation (38). In some cases, an indirect immunofluorescence assay was used to confirm the presence of BIV. For these assays, cells were fixed in 70% acetone–30% methanol and incubated for 30 min at room temperature with 0.5 ml of a predetermined dilution of BIV-specific antibody in Hanks balanced salt solution. The polyclonal BIV-specific antibody, obtained from a calf experimentally inoculated with BIV, was free of antibody to BVDV, BLV, and bovine spumavirus. Following incubation with antibody, cells were washed twice in Hanks balanced salt solution and incubated with a predetermined dilution of fluoresceinated anti-bovine immunoglobulin G antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa.). Cells were incubated, washed as described above, and examined for BIV-specific immunofluorescence. All cultures were passaged at least three times before they were considered virus negative.

**Antibody detection.** Serum samples collected from experimentally inoculated calves at 5 to 6 weeks p.i. were tested for antibodies to BIV by Western immunoblot assays as previously described (41, 42). A focal immunofluorescence assay (14, 15, 35) was used for detection of BVDV-specific antibody. A total of 5 × 10⁵ BVDV-free MDBK cells were seeded in 60-mm tissue culture dishes, and 10³ MDBK cells chronically infected with noncytopathic BVDV were added the following day. After 4 days, cells were fixed as described above and incubated with serial twofold dilutions of sera collected from experimentally infected and control calves. Positive control sera included convalescent sera from a BVDV-infected calf. Following 30 min of incubation at room temperature, cells were washed, treated with secondary antibody, and examined for BIV-specific immunofluorescence.

**In situ hybridization.** All enzymes, isotypes, vectors, and other chemicals were from sources previously detailed (1–3, 5, 9). The complete 587-nucleotide sequence of the BIV long terminal repeat (LTR) was amplified by using the polymerase chain reaction (PCR) and cloned as follows. Oligonucleotide primers spanning nucleotides 1 to 20 of the 5’ end of the U3 region and complementary to nucleotides 570 to 587 in the 3’ end of the U5 region were designed on the basis of the published sequence of BIV (19). Each of these primers included eight additional 5’ nucleotides containing a HindIII site. Two primers complementary to sequences in the central region were also synthesized. Total DNA was extracted from MDBK cells chronically infected with BIV R29, and 100 ng of DNA was amplified for 25 cycles, using the LTR terminal primers and PCR conditions as previously described (4, 13). The PCR-amplified products were partially purified by phenol-chloroform extraction, digested with HindIII, and ligated into the HindIII site of pUC19. Colonies were blotted onto nylon membranes and probed with the internal LTR oligonucleotides 5’ end labelled with ³²P as previously described (4, 13). Positive colonies were picked and replated twice. A single positive clone, designated 4-N#1.6, was further characterized by restriction enzyme
mapping and deoxy DNA nucleotide sequencing as we previously described (1, 8, 13). The nucleotide sequence of 4-N#1.6 was more than 99% homologous to the sequences previously reported (19).

For use in strand-specific in situ hybridization, the BIV LTR was subcloned in the HindIII site of pGEM4Z (Promega Biotec, Madison, Wis.). Clones containing a single BIV LTR insert in the forward orientation with respect to the T7 promoter (clone 5-H#11.2, producing a plus-sense RNA transcript) and in the reverse orientation (clone 5-H#11.3, producing a minus-sense transcript) were identified. For use as probes, RNA from plasmids 5-H#11.2 and 5-H#11.3 was linearized to completion with SmaI and transcribed in vitro under conditions previously detailed (3, 9). The RNA was radiolabelled with [35S]UTP for filter blot hybridizations and with [35S]UTP for in situ hybridization. The size of the transcripts was verified by electrophoresis of a portion of the labelled RNAs into a formaldehyde-agarose gel and comparison with known standards. Approximately 100 to 200 ng of RNA transcript of about 600 nucleotides in length and a specific activity of $3 \times 10^6$ cpm/µg could be produced from 200 ng of plasmid DNA. The sense specificity of the probes was tested by hybridization to replicate Northern (RNA) blots of total RNA isolated from BIV-infected MDBK cells (1, 3, 9). BIV RNA reacted with the minus-sense probe but not with the plus-sense probe (data not shown). For a detailed discussion of strand-specific probes, the reader is referred to previous reports (1–3, 5, 7, 9, 37).

In the in situ hybridizations were performed on periodate-lysine-paraformaldehyde-glutaraldehyde-fixed cells and tissues as previously described (2, 3, 5, 7, 37) except that the BIV probes were used and the temperature of stringent wash was lowered to 50°C. The LTR sequences were chosen as probes in order to rule out any possible cross-hybridization to BLV, as the BIV and BLV LTRS are only 40% related at the nucleotide level (19, 34). RNA probes were chosen because they give excellent sensitivity and specificity due to the posthybridization RNase step.

RESULTS

Clinical and hematological changes following experimental inoculation of calves with BIV. We undertook a series of experiments to characterize the in vivo virulence of BIV R29 during the first 6 weeks after infection and to determine whether viral virulence could be increased following rapid in vivo passage. Following inoculation, calves were examined at least three times per week for changes in temperature and hematological profiles. No hematological values outside the normal range were observed in the p1 calf inoculated with BESP cell culture-derived R29 (Fig. 1). Multiple transient episodes of neutropenia were observed in the p2 through p4 calves. In these cases, an initial period of neutropenia occurred at 5 to 15 days p.i., followed by a second cycle of neutropenia at approximately 4 weeks p.i. The lack of the initial period of neutropenia in the p5 calf may be due to the development of pneumonia in this calf at 3 days p.i. At that time, the calf was treated with antibiotic for 2 days, and it quickly recovered. All other animals remained clinically normal throughout the experimental period. Increased numbers of lymphocytes were observed in several BIV R29 passage calves, notably the p2 and p5 calves. However, the lymphocyte values were all within the normal range. Isolated days of fever occurred in the p2 and p3 calves, while a more extended febrile response, most likely associated with pneumonia, was observed in the p5 calf.

In a second series of experiments, we passaged the molecularly cloned BIV 106 isolate through three calves. BIV 106 is an R29-derived molecular clone of BIV previously shown to be infectious for bovine cells in vitro (12). Cell-free stocks of BIV 106 were inoculated directly into a Holstein calf and passaged three times in vivo as described above. Following inoculation, the BIV 106 p1 calf experienced a prolonged febrile response and severe neutropenia (Fig. 1). Clinical signs included anorexia, lethargy, ataxia, and diarrhea. The BIV 106 p2 calf experienced milder fever and neutropenia, while the p3 calf was intermediate in both parameters. No other clinical signs or hematological changes were observed in either the p2 or p3 calf.

Fever and leukopenia are characteristic of acute infection with BVDV (23, 32, 36). BVDV is a common contaminant of cell culture material (10) and, in fact, is present in all stocks of BIV which we have examined (data not shown). Therefore, additional experiments were undertaken to clarify the respective roles of BIV and BVDV in the inocula. Seven colostrom-deprived calves seronegative for BLV and BVDV were vaccinated with killed BVDV vaccine at 2 and 4 weeks prior to inoculation with BIV. Four calves were inoculated with 50 ml of FBL containing $1.8 \times 10^3$ syncytium-forming units of BIV R29. The remaining three calves received 50 ml of BIV-free FBL cells. Noncytopathic BVDV was present in all inocula. Calves were monitored for changes in temperature and hematological profile as described above.

FBL-BIV R29-infected calves had significantly higher numbers of circulating lymphocytes than did the FBL controls during the first 6 weeks p.i. The mean lymphocyte count in the BIV-inoculated animals during the first 6 weeks p.i. (5,940 cells per µl), was significantly higher ($P < 0.02$) than the mean of the control group (4,093 cells per µl). In addition, two of the four BIV-inoculated calves had lymphocyte counts above the normal range (Fig. 2). This was in marked contrast to the control calves, for which lymphocyte counts were often observed to be below the normal range, indicative of the lymphopenic effects of BVDV (33). BVDV-associated neutropenia was observed in both groups of calves. The mean number of neutrophils in the FBL-BIV R29-infected calves was lower than in the FBL controls; however, the means were not significantly different. Both groups of calves experienced transient fever during the first 2 weeks p.i., and no differences were observed between the groups in the severity or duration of fever (data not shown). Thus, the only hematological effect that could be directly attributed to BIV was an increase in the numbers of circulating lymphocytes.

Virus isolation and serological response. Whole blood was collected from each calf at least twice during the experimental period and tested for the presence of infectious BIV and antibody to BIV and BVDV. BIV was recovered from all of the R29 passage calves at 2 weeks p.i., and BIV-specific antibody was detectable in all p.i. sera from calves inoculated with BIV R29 (Table 1; Fig. 3). The strongest serological response to BIV was observed in the p2 and p5 calves (Fig. 3). No consistent, progressive increases in antibody titer or protein specificity were identified in the BIV R29 passage calves which could be correlated with successive passage of BIV. These results suggested that in vivo passage of BIV R29 did not dramatically alter levels of virus replication in vivo.

In contrast to the BIV R29 passage calves, BIV was not recovered from BIV 106-inoculated calves (Table 1). Virus could not be reisolated from the p1 calf during the prolonged febrile period at days 7 through 14 p.i. Therefore, it could not
be demonstrated that BIV was present in the whole blood inoculum transfused into the p2 calf. In addition, BIV-specific antibody was detectable only in the BIV 106 p1 calf, while the p2 and p3 calves remained seronegative throughout the experimental period (Fig. 3). The lack of BIV-specific antibody in the p2 and p3 calves, and the failure to recover virus from the p1 calf early after experimental infection, suggested that the BIV 106 inoculum replicated poorly in vivo. Importantly, these results indicated that the fever and neutropenia observed in the BIV 106 p2 and p3 calves were not likely due to BIV replication. In the third experiment, we were able to isolate BIV from PBMC collected at 2 and 6 weeks p.i. from all FBL-BIV R29 calves but from none of the FBL controls. The serological response of FBL-BIV R29 calves was, in general, stronger than that observed in BIV R29 passage calves, especially to the viral envelope glycoprotein, gp110 (41, 42).

Results of BVDV serology indicated that none of the calves in these experiments were infected with BVDV prior to BIV inoculation (data not shown). However, the BIV R29 p1 through p3 calves and all of the BIV 106 passage calves seroconverted to BVDV within 6 weeks p.i. (Table 1). A rapid secondary antibody response was detected in all BVDV-vaccinated calves inoculated with FBL-BIV R29 (data not shown). Together, these results confirmed that both the BIV R29 stock and the BIV 106 cell-free stock contained infectious BVDV. Interestingly, the BIV R29 p4 and p5 calves did not seroconvert to BVDV by 5 to 6 weeks p.i., although both had seroconverted to BIV in a similar time frame. This serological evidence indicated that the p4 and p5 calves were BVDV free and therefore suggested that the cycles of neutropenia observed in the p4 calf were a consequence of BIV infection.

Pathological changes in BIV-infected calves. At 5 to 6 weeks p.i., BIV R29 passage calves were euthanized and examined for gross and histopathological changes. Grossly, lymph
times three indicated weeks experimental inoculation. All cultures were passaged using BIV-specific antibody. Sera BIV age-matched, noninoculated animals. FBL 356 FBL-R29 340 R29 106 BIV Inoculum Calf BVDV BIV e PBMC Calves experimentally a inoculated animals. FIG. 2. Numbers of circulating lymphocytes and neutrophils in peripheral blood during the first 6 weeks following experimental inoculation of calves with FBL-BIV R29 or FBL control cells. Shaded areas indicate 95% of lymphocyte or neutrophil values obtained from age-matched, noninoculated animals. Individual animals are represented by different symbols within each panel.

nodes often had slight to moderate swelling seen as bulging of the parenchyma when the capsule was incised. Infrequently, entire lymph nodes or portions of them were hyperemic. Hemal nodes were pink to bright red and ranged from 2 mm to 1.5 cm in diameter. Consistent histologic changes characterized as lymphoid follicular hyperplasia were found in lymph nodes, hemal nodes, spleen, tonsil, Peyer's patches, and other gut-associated lymphoid tissue in infected calves. Sections of brain, spinal cord, salivary gland, esophagus, trachea, lung, thymus, heart, liver, pancreas, adrenal, kidney, abomasum, small intestine, large intestine, and bone marrow were normal.

The most frequent histopathological finding was lymphoid hyperplasia, primarily due to expansion of the number and size of follicles (Fig. 4). Both primary and secondary follicles were present in lymphoid tissues of the control calf; however, secondary follicles were more numerous in BIV-infected calves. In the BIV-infected calves, the secondary follicles occupied most of the subcapsular cortical space and were frequently observed in the deep cortex, especially in areas adjacent to fibrous trabeculae. Cells in the center of hyperplastic follicles were larger and generally less identifiable as histiocytic or lymphocytic types with the exception of macrophages containing cytoplasmic tingible bodies. In the paracortex, scattered large pale histiocytic cells and occasional mitotic figures were present among the predominating large and small lymphocytes. In hemal nodes, moderate to marked follicular hyperplasia was commonly observed and was similar to that observed in the lymph nodes.

The degree of lymphofollicular hyperplasia was variable among the lymph nodes and hemal nodes from different anatomic locations and between nodes and other lymphoid organs within an infected animal. The degree of hyperplasia also varied among the infected animals; however, patholog-

### TABLE 1. Detection of virus and antibody in experimentally inoculated calves

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Calf</th>
<th>Presence of BIV</th>
<th>BIV antibody</th>
<th>BVDV antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIV R29</td>
<td>p1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>p2</td>
<td>+</td>
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<tr>
<td></td>
<td>p5</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
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<td>ND</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
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<tr>
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<td>-</td>
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<td>+</td>
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<td></td>
<td>359</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</table>

* Calves experimentally inoculated with either BIV R29, BIV 106, FBL-BIV R29, or FBL control cells were bled 2 to 3 weeks p.i., and isolated PBMC were tested for the presence of BIV by cocultivation with BESP or FBL cells. Sera were collected prior to inoculation and at 5 to 6 weeks p.i. and tested for antibody to BIV and to BVDV.

* PBMC (10^7) were isolated from experimentally inoculated calves at 2 to 3 weeks p.i. and cocultivated with BESP or FBL cells. The presence of BIV was indicated by the appearance of syncytiaind and, in some cases, by indirect immunofluorescence using BIV-specific antibody. All cultures were passaged three times before they were considered negative. ND, not determined.

* BIV seroconversion was determined by Western immunoblot.

* BVDV seroconversion was detected by indirect immunofluorescence.

* Calves were vaccinated with killed BVDV vaccine at 2 and 4 weeks prior to experimental inoculation.
logical changes did not increase in severity with progressive passage of BIV, nor could differences be attributed to the presence or absence of concurrent BVDV infection.

In situ hybridization. To determine sites of BIV replication in vivo, certain tissues, including spleen, lymph node, liver, lung, and PBMC, were analyzed by in situ hybridization. BIV-specific RNA probes were prepared as described above and initially tested for specificity and sensitivity, using cytospin preparations of BIV-infected and non-infected MDBK cells. No hybridization signals above background were observed with use of the plus-sense probe, indicating that the levels of BIV DNA were low in all in vitro and in vivo samples tested. With the minus-sense probe, however, positive hybridization signals representing BIV mRNA and/or virion RNA were detectable. BIV RNA could be detected in cell culture material after only 3 days of exposure of the autoradiographic emulsion, and after 18 days, heavy grain formation was observed (Fig. 5). In contrast, relatively few grains were present in PBMC from BIV-infected calves after 1 month of exposure. Thus, the levels of BIV RNA in virus-infected cells in vivo were at least 10-fold lower than RNA levels detected in vitro cell culture. In addition to the relatively low levels of virus expression per cell, the number of BIV-infected PBMC was found to be less than 25/10^5 cells (Table 2). Analysis of tissue from BIV R29 passage calves identified only a rare BIV-positive cell in the spleen, liver, and lymph node connective tissue (data not shown). However, no evidence of BIV replication could be detected in lymph node germinal centers.

DISCUSSION

BIV was originally isolated from a cow with persistent lymphocytosis (38). In the original studies of BIV pathogenesis, VanDerMaaten et al. reported that calves inoculated with BIV developed a mild lymphocytosis and moderate lymphoproliferation in the small subcutaneous lymphatic nodules (38). The virus was found to be morphologically similar to visna virus (11, 38) and was later demonstrated to have antigenic and genetic similarity to HIV (19, 21). The structural similarities between BIV and HIV, as well as the presence of lymphadenopathy in BIV-infected calves, indicated that BIV may provide a useful animal model of the pathogenesis of HIV infection (21). In the present study, experimental infection of calves with BIV was found to induce mild lymphoproliferative changes within the first 6 weeks following infection. These changes included follicular hyperplasia in lymph nodes, hemal nodes, and spleen as well as increased numbers of lymphocytes in peripheral blood.

Histopathological changes in BIV-infected calves consisted of follicular hyperplasia in lymphoid tissues. The extent of hyperplasia was variable among the infected animals; however, all had secondary follicles in which proliferation of follicle center cells had progressed to impinge on the mantle zone of small lymphocytes. These changes are similar to early-stage lymph node alterations in patients with HIV-1 infections (16, 20, 30). Thus, follicular hyperplasia without follicular fragmentation characterizes both the early response to BIV infection in calves as well as the early response to HIV-1 infections in humans. Similar lymphoid changes have also been described for SIV (17) and FIV (44). In both HIV and SIV infections, follicular hyperplasia is due in part to the formation of antigen-antibody complexes and antigen trapping within the lymphoid follicle (31, 43). Antigen-antibody complexes may also be involved in BIV-induced follicular hyperplasia, as all animals had seroconverted to BIV prior to postmortem examination. In addition,
FIG. 4. Pathological changes in lymph nodes and hemal nodes following experimental inoculation of BIV R29. (a) Subcapsular primary follicle in the lymph node of a control calf. Note the lack of a mantle zone of small lymphocytes surrounding the follicle center (arrowhead). H&E stain, ×80. (b) Higher magnification of control lymph node showing unstimulated follicle center cells and moderate numbers of small lymphocytes and larger histiocytic cells with abundant pale cytoplasm and vesicular nuclei. H&E stain, ×160. (c) Lymph node from the BIV R29 p5 calf taken at 6 weeks p.i. Note the increased number of secondary follicles in both the subcapsular and deep cortex (arrowheads). H&E stain, ×80. (d) Lymph node secondary follicle from the BIV R29 p5 calf showing proliferation of follicle center cells, scattered histiocytes with stainable granular debris in the cytoplasm, shrunken cells with pyknotic or fragmented nuclei (apoptotic cells), and a mantle of small lymphocytes. H&E stain, ×160. (e) Hemal node from the BIV R29 p1 calf at 6 weeks p.i. showing follicular hyperplasia. One secondary follicle and portions of two others are indicated by arrowheads. H&E stain, ×80. (f) Higher magnification of panel e showing follicular changes similar to those in panel d. H&E stain, ×160.

we were unable to detect BIV-infected cells in germinal centers of hyperplastic follicles by in situ hybridization, suggesting that hyperplasia was not a direct result of virus replication within the germinal center. These findings are similar to those reported for in vivo infection with SIV, where few virus-infected cells are detectable in lymph node germinal centers during stages of follicular hyperplasia (43). Interestingly, the number of SIV-infected cells increases during later stages of follicular depletion (43). At present, it is not known whether degenerative changes in lymphoid
TABLE 2. Quantitation of BIV-infected PBMC by in situ hybridization

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Calf</th>
<th>Positive cells/10⁶ PBMC&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>FBL-BIV R29</td>
<td>340</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>341</td>
<td>5</td>
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<sup>a</sup> PBMC were isolated from whole blood at 6 weeks p.i. from calves experimentally inoculated with FBL-BIV R29 or with FBL control cells; 10⁵ cells were cytocentrifuged onto poly-D-lysine-coated slides and hybridized with a minus-sense BIV LTR probe as described in Materials and Methods.

<sup>b</sup> Cells were scored as positive when the number of grains over an individual cell was at least fivefold higher than the number of grains over uninfected cells.

follicles associated with viral replication within the germinal center are characteristic features of a later stage of BIV infection in vivo.

In addition to the mild lymphoproliferative changes, transient periods of fever and neutropenia were observed in experimentally infected calves. These findings are similar to those reported for experimental infection of cats with FIV (44) and in acute stages of HIV infection (18). However, acute BVDV infection of cattle is characterized by biphasic febrile episodes and neutropenia during the first 2 weeks following infection (23, 32, 36). Because BVDV was present in almost all of the BIV-inoculated calves, it was not always possible to determine whether clinical changes were due to BVDV, BIV, or both.

The R29 stock of BIV has been shown to contain mixtures of viral genotypes; however, the predominant virus genotype appears to be BIV 106-like (12). Thus, it is interesting to note that although R29 could be readily transmitted at 2 weeks p.i., we were unable to transmit the molecular clone BIV 106. These results may indicate that the BIV 106 molecular clone replicates poorly in vivo and that other genotypes better suited to in vivo replication may be present in the R29 stock. However, the differences in vivo replication may be more characteristic of the particular inoculum used in these experiments. The R29 p31 calf was inoculated with virus-infected cells and culture supernatant, while the BIV 106 p1 calf was inoculated with cell-free virus. Studies in our laboratory indicate that in vitro transmission of BIV occurs more readily if the inocula contain infected cells rather than cell-free virus. Ongoing studies to characterize isolates recovered during in vivo passage should provide a better understanding of the biological and genetic phenotypes present in the R29 stock of BIV.

In this study, the extent of the clinical and pathological changes following experimental BIV infection was less pronounced than was described for naturally infected cattle (38). These results are consistent with previous studies of experimental BIV infections (38, 39) in which the magnitude of lymphocytosis was considerably less than that observed in the naturally infected cattle from which BIV was originally isolated (38). A variety of factors may contribute to the observed differences between naturally infected and experimentally infected animals. One factor is the time required for the clinical manifestations of disease to appear. The original BIV-infected cow, R29, was 8 years of age at the time of BIV isolation, and in subsequent experimental infec-

FIG. 5. In situ hybridization for detection of BIV-infected cells. Cells (10⁵) were cytocentrifuged onto poly-D-lysine-coated slides and hybridized with a minus-sense BIV LTR probe as described in Materials and Methods. (a) PBMC taken 6 weeks p.i. from a calf inoculated with FBL control cells. After 1 month of exposure, only background grains are seen. (b) PBMC taken 6 weeks p.i. from a calf inoculated with FBL-BIV R29. After 1 month of exposure, a significant number of grains representing hybridization to BIV RNA are seen over the nucleus and cytoplasm of an infected cell. (c) MDBK cells exposed for 18 days. Only background numbers of grains are observed. (d) MDBK cells chronically infected with BIV R29. After 18 days of exposure, very high numbers of grains are seen over an infected cell.
ions, palpable hemal nodes were not detected until 3 months postinfection (38). A second factor is the possibility that BIV virulence depends on the presence of additional cofactors present in the naturally infected animal. BLV is endemic in many parts of the southern United States, and coinfection of cattle with BIV and BLV could result in enhanced lymphoproliferation. BLV had not been identified as the causative agent of bovine lymphosarcoma at the time of the original isolation of BIV, and it is not known whether cattle from which BIV was isolated were in fact coinfected with BLV. A third factor to consider is that the current stocks of BIV may have lost virulence as a result of long-term in vitro culture. We have shown that in vitro culture can rapidly select for lentivirus isolates with altered biological phenotypes, including decreased virulence (14). In this study, we attempted to increase viral virulence by rapid in vivo passage. Although similar strategies have proved successful with other lentiviruses (29), we observed no increase in levels of virus replication, serological response, or clinical and pathological changes which specifically correlated with successive in vivo passage. Therefore, it is not clear whether low virulence is a biological phenotype of the R29 stock or, rather, is representative of all isolates of BIV. Identification and characterization of additional field isolates of BIV are needed to increase our knowledge of BIV pathogenesis.

In summary, the results of this study indicate that the pathological changes during the early periods following experimental infection of calves with BIV are characteristic of several immunosuppressive lentiviruses, including HIV, SIV, and FIV. Together with the genetic and antigenic relatedness of these viruses, the in vivo studies suggest that BIV is a useful animal model for the study of the early events in HIV pathogenesis. Ongoing studies to define the long-term pathogenic effects of BIV infection in vivo will determine the appropriateness of BIV as an animal model of HIV-associated immune deficiency syndrome.

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