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Isolation and Characterization of New Wild-Type Isolates of Bovine Lentivirus

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Two new isolates of bovine lentivirus, also known as bovine immunodeficiency-like virus (BIV), were obtained from a seropositive cattle herd in Florida. This is the first report of new isolates of BIV since the original BIV strain, R29, was isolated in 1969. The two new BIV isolates were derived from blood buffy coat cells cocultivated in vitro with fetal bovine lung cell cultures. The new isolates differed in vitro from the original R29 isolate in replication and syncytium formation in fetal bovine lung cells. Both new isolates were confirmed as BIV by immunofluorescence assay, Western blotting (immunoblotting), and polymerase chain reaction. Sequence analyses of the polymerase chain reaction *pol* gene product showed 92.6 and 93.6% homology to the published nucleotide sequence of BIV R29-127, a molecular clone derived from BIV R29. Each of the new BIV isolates was inoculated into two calves, and virus was recovered between 5 and 10 days postinoculation (p.i.), with BIV seroconversion between 10 and 21 days p.i. Virus was recoverable and antibody was detectable for at least 4 months p.i. Two calves developed a transiently elevated mononuclear cell count, similar to what was reported for BIV R29 in the original experimental calf inoculations. No other clinical abnormalities were observed.

The original isolation of the bovine immunodeficiency-like virus (BIV) was made in 1969 from an 8-year-old dairy cow from Louisiana that had a persistent lymphocytosis and was becoming progressively emaciated. At the time of necropsy, this cow had clinical and histopathological lesions, including perivascular cuffing in some vessels in the brain and enlarged lymph nodes and hemal nodes (28). The viral isolate from this dairy cow, designated R29, was originally described as a visna-like virus because of similarities to the ovine lentivirus. With the finding that human immunodeficiency virus type 1 (HIV-1) is a lentivirus, renewed interest in the bovine visna-like virus resulted in its molecular characterization, which conclusively placed BIV in the lentivirus family as a unique member of the group (4, 10, 11).

BIV causes a persistent infection in cattle, and serological data indicate that it may have a worldwide prevalence (2, 3, 13, 14, 29, 30). BIV has a genomic organization similar to that of other lentiviruses, including the *pol*, *gag*, *env*, and *tat* genes (10, 24). Antigenically, antiserum to BIV p26 cross-reacts with p24 of HIV-1 and antiserum to equine infectious anemia virus cross-reacts with p26 and p24 of BIV (11, 31). Two infectious molecular clones of BIV R29, BIV R29-127 and BIV R29-106 have been sequenced, showing that the virus is a unique member of the lentivirus family (4, 10). BIV R29-106, however, did not replicate well in cattle after experimental inoculation (6).

All molecular characterizations and animal inoculation studies of BIV have been done with the original BIV R29 isolate, which has been passaged extensively in vitro or frozen for over 20 years, and it may be attenuated (6, 8, 10). The R29 isolate has also become contaminated with a noncytopathic strain of bovine viral diarrhea virus (BVDV). The original experimental inoculations of BIV R29 in colostrum-deprived calves induced mild lymphocytosis and enlargement of peripheral subcutaneous lymph nodes, with no overt clinical signs of disease (28). In recent studies with (i) BIV R29-1203 inoculant free of BVDV, (ii) R29 inoculant in BVDV-vaccinated cattle, or (iii) BIV R29-106 molecular clone inoculant, the original experimental findings of leukocytosis and lymph node enlargement have not been observed, suggesting that the virus has become attenuated (6, 10). Attenuation of lentiviruses in cell cultures has been described in detail for equine infectious anemia virus (5, 25). However, the virulence of one equine infectious anemia virus isolate was increased with rapid animal passage (25). Similar experiments with BIV have not resulted in a change in the course of infection. The BIV R29-106 molecular clone, which is infectious for cell cultures in vitro, was not sustained by animal passage through whole-blood transfer (6, 10).

Several studies with BIV have explored the possibility that this virus causes immune system alterations, similar to HIV-1 or feline immunodeficiency virus. However, because the original BIV isolate, R29, has become contaminated with a noncytopathic strain of BVDV, an RNA virus that can cause immune suppression in bovines, interpretation of data acquired from cattle inoculated with this isolate has become complicated (6, 21). Studies examining immune function of BIV in vivo have demonstrated either mild or no immunosuppression on the basis of lymphocyte blastogenesis tests, neutrophil function tests, mononuclear subset analysis, and histopathological changes (6, 8, 21). Available research results have not shown that BIV is a disease-causing agent in cattle in experimental inoculations. These reports were all based on studies of cattle infected for less than 27 months (6, 8, 21, 28–30). Thus, the possibility that BIV has a long incubation period before causing disease or that it has the ability to act as a cofactor in disease cannot been ruled out (6, 29).

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In this report, we describe the in vitro and in vivo characterization of two new wild-type isolates of BIV which were isolated from two cows in a seropositive dairy herd in Florida. A BVDV-free BIV isolate of R29, BIV R29-1203, was also used in this study as a positive control to help contrast the differences between the new isolates and R29-derived isolates. The R29-1203 isolate was prepared by vaccinating a BIV-BVDV-negative calf with an autogenous BVDV vaccine prepared (S. R. Bolin, National Animal Disease Center, Ames, Iowa) from a clone of the noncytopathic BVDV isolated from BIV R29 cell cultures. After the calf developed neutralizing antibodies to BVDV, it was inoculated with BIV R29. BIV was recovered from blood buffy coat cells, tested both in vitro by cell culture methods and in vivo by calf inoculation, found to be free of BVDV, and designated BIV R29-1203.

In vitro characterization of BIV isolates. A Florida dairy herd with BIV-seropositive cattle was identified by Western blot (immunoblot) assay, and blood samples in EDTA tubes were taken for virus isolation (31). The blood samples were centrifuged, and the buffy coat cells were removed for coculture with fetal bovine lung cells cultured in Eagle's minimum essential medium with Earle's salts supplemented with 10% fetal bovine serum, Polybrene (4 μg/ml), gentamicin (50 μg/ml), and 0.02% L-glutamine. These cultures were blind passaged and/or cultured with fresh fetal bovine lung cell cultures until the cytopathic effects of syncytium formation and cell lysis were observed. Evidence of viral replication by syncytium formation was observed in two cultures, FL491 and FL112, after the fourth blind passage in culture. The two cows from which the isolates were derived were seropositive for BIV on Western blot assay and seropositive for bovine leukemia virus (BLV) and seronegative for bovine syncytial virus (BSV) on agar gel immunodiffusion (AGID) (19, 22, 31). Continued culturing of FL491 and FL112 gave increased syncytium formation with limited cell lysis. The new isolates showed differences in replication characteristics in vitro compared with BIV R29, including higher cocultivation ratios, longer incubation times between passages, and differences in syncytial appearance typified by a decreased number of nuclei per syncytium and greater granularity and slower lysis of the syncytia compared with BIV R29 and BIV R29-derived isolates. Syncytial nuclei from the new wild-type isolates often formed a circular pattern in culture which was not commonly observed in BIV R29-derived cultures. The BIV R29-1203 isolate demonstrated higher cytopathogenicity in cell culture than did the new isolates. The FL491 and FL112 wild-type isolates had similar replication patterns in vitro with smaller syncytia and a less lytic effect on cell cultures. The replication characteristics of the different BIV isolates were maintained in relation to their parent strain after animal inoculation.

The new wild-type isolates of BIV have biological differences in comparison with R29 in vitro, and in vitro characteristics of other lentiviruses have been shown to correlate with virulence in vivo (7). Specifically, the more cytopathogenic lentiviral strains in culture are associated with greater in vivo virulence in HIV-1 and greater lymphoproliferative change in the ovine lentivirus maedi-visna virus (7, 12, 16, 17). Different regions of the viral genomes have been associated with these in vitro and in vivo characteristics (9, 12, 23, 32). Biological variation should not be unexpected among BIV isolates, and these differences are probably controlled by several different areas of the genome.

FL491 and FL112 were BIV positive by immunofluorescence assay using BIV-specific antiserum and fluorescein-conjugated rabbit anti-bovine immunoglobulin G, but both isolates were indirect immunofluorescence assay negative for BLV and BSV by the same technique (30). Western blot assays using viral antigen prepared as previously described (31) from each of the isolates with positive reference control sera were positive for BIV, negative for BLV, and negative for BSV. AGID tests using positive reference sera for BLV and BSV were negative (data not shown) (19, 22). DNA samples for polymerase chain reactions (PCR) were prepared from BIV-infected fetal bovine lung cell cultures with proteinase K followed by phenol-chloroform extraction by standard procedures (20). One microgram of template DNA, 20 pmol of primer, and 1.25 U of *Taq* polymerase (Cetus Corp., Norwalk, Conn.) in a 50-μl reaction mixture were used in a standard PCR mixture with first-cycle conditions of 94°C for 2 min, 51°C for 15 s, and 72°C for 2 min and then 30 cycles of 94°C for 45 s, 51°C for 15 s, and 72°C for 1 min and a final extension step of 72°C for 10 min. The *pol* gene plus-sense primer 5′ ATG CTA ATG GAT TTT AGG GA 3′ and the minus-sense primer 5′ CAT CCT TGT GGT AGA ACA TT 3′ amplified a 242-bp product from BIV R29-1203-, FL491-, and FL112-infected cell cultures (Fig. 1) whose size was predicted by published sequence data and previously reported work (10, 15). Southern blot analyses with the BIV *pol* gene-specific probe confirmed the identities of the PCR products (20). Each PCR product was then directly sequenced by purification with a Centricon 100 microconcentrator and use of 40 ng of DNA with *Taq* polymerase in a dye terminator sequencing system on an automated sequencer (ABI Applied Biochemistry 373A DNA Sequencer; performed at the Nucleic Acid Center, Iowa State University, Ames). Nucleotide sequences were analyzed for homology by using the Wilbur-Lipman methods (DNASTAR program Align). The 212-bp sequenced PCR products were compared to the published sequence for the putative reverse transcriptase gene segment of BIV R29-derived molecular clone BIV R29-127 (10). The BIV R29-1203 isolate had 99.6% nucleotide sequence homology with the published sequence, with only a single, silent nucleotide substitution. The BIV FL491 and FL112 isolates had 93.6 and 92.6% nucleotide sequence homology, respectively, to the published BIV sequence and 96% nucleotide sequence homology with each other. All of the sequenced products maintained the same open reading frame for a segment of the putative reverse

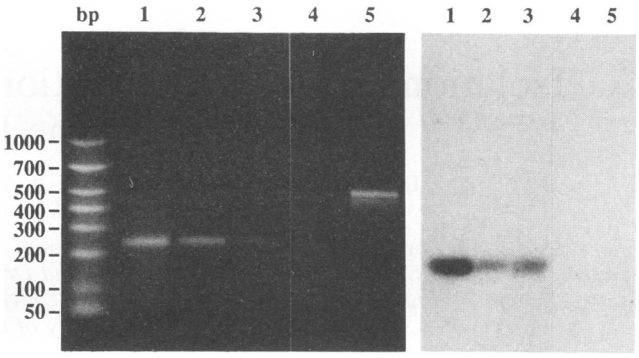

FIG. 1. Products of PCR amplification (left) of a 242-bp segment of the *pol* gene from BIV R29-1203 (lanes 1), BIV FL491 (lanes 2), and BIV FL112 (lanes 3) and Southern blot hybridization (right) of those products with a ^{32}P-labeled *pol* gene probe. Negative (lanes 4) and positive (lanes 5) PCR controls and a molecular size ladder are also shown.

transcriptase gene, and no insertions or deletions were seen in the sequenced product. The predicted amino acid homologies for BIV FL491 and FL112 were 93.8 and 92.3%, respectively.

The reverse transcriptase region of the genome is highly conserved among other lentiviruses, and it is expected that BIV is similar. Comparison of the *pol* gene region of the BIV R29-1203 isolate, which has been through an animal passage and over 35 passages in cell culture, to the published sequence for BIV R29-127, a molecular clone of BIV R29, demonstrated that BIV R29-1203 has only a single, silent nucleotide change from the published sequence. This suggests that this segment of the reverse transcriptase region may be highly conserved in BIV and provides a valuable guidepost for comparison of new isolates. The new Florida isolates have 7 to 8% nucleotide sequence divergence in the conserved *pol* segment compared with Louisiana BIV R29-127, demonstrating the divergence of these isolates from R29. The new isolates have only 4% nucleotide sequence divergence between them, suggesting that they are more closely related field isolates. Comparisons of different HIV-1, visna virus, and feline immunodeficiency virus isolates show that the diversity of nucleotide sequences of isolates generally is greatest when they are from geographically distinct populations (1, 18, 26, 27). In comparison with these three lentivirus groups, the new Florida BIV isolates appear to be distinct from the Louisiana isolate but are not as divergent as other geographically distinct lentivirus isolates. Sequencing of other BIV isolates will help to determine whether the Florida isolates are distinct or continuums of the same BIV population.

In vivo characterization of BIV isolates. Four calves negative for antibodies to BIV, BLV, and BSV were experimentally inoculated with the FL491 and FL112 isolates. Each calf received BIV-infected fetal bovine lung cells and culture fluids intravenously in the jugular vein and subcutaneously in the area around the jugular vein as follows: BIV FL491, Jersey calf 1268 and Holstein calf 848; BIV FL112, Jersey calves 1275 and 1269. Calves were observed for clinical symptoms, and blood samples were taken at 3, 5, 7, 10, 14, 21, and 28 days postinoculation (p.i.) for virus isolation, serology, and leukocyte (WBC) differential counts. Thereafter, the calves were sampled weekly for serologic and WBC responses and every 2 to 4 weeks for virus isolation.

All four calves developed BIV-specific antibody responses as determined by Western blot analysis. Results of Western blotting of serum samples from calf 1275 taken preinoculation, on p.i. day 35, and on p.i. day 91 and from calf 1268 taken preinoculation, on p.i. day 35, and on p.i. day 105 are shown in Fig. 2B. Calves 1268 and 848, inoculated with BIV FL491, developed a BIV-specific serological response starting at p.i. days 10 and 20, and virus was recovered from samples starting on p.i. days 10 and 11, respectively. Calves 1275 and 1269, inoculated with BIV FL112, both developed a BIV-specific serological response starting at p.i. day 14, and virus was isolated from samples by p.i. days 5 and 7, respectively. Viral antigens prepared from virus recovered from experimentally inoculated calves 1268 and 1275 were used in a Western blot (Fig. 2A) and were positive with BIV reference sera. Western blot antigens were also tested with both BLV and BSV reference sera and were negative (data not shown).

The WBC response for calves 1275 and 1269, inoculated with FL112, showed transient leukocytosis consisting mainly of mononuclear cells (Fig. 3). The WBC response for calves 1268 and 848, inoculated with FL491, was slightly

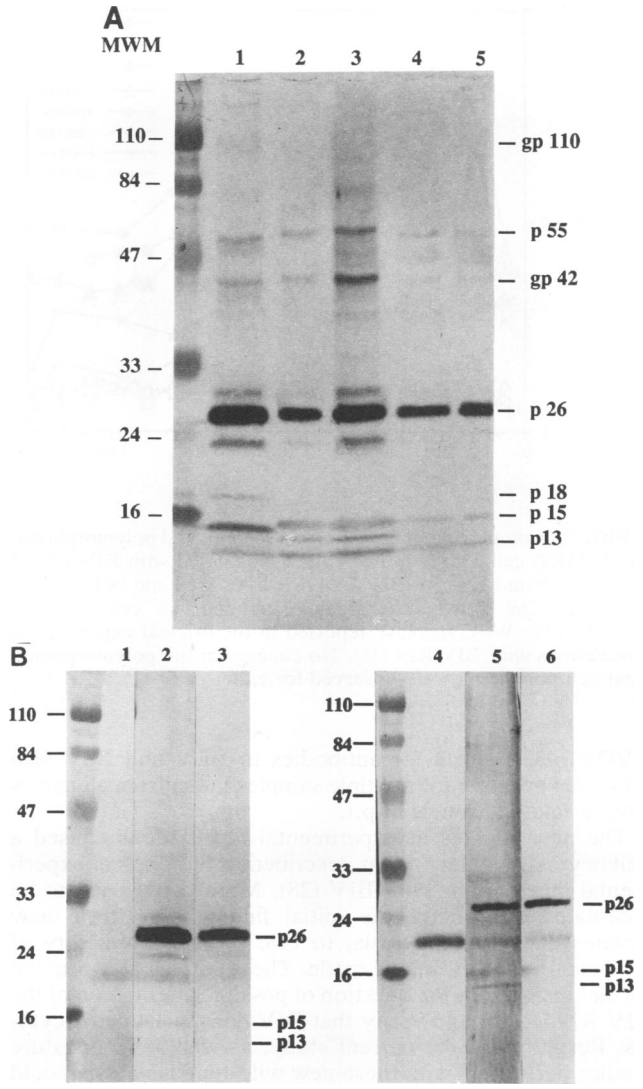

FIG. 2. (A) Western blot assay in which BIV reference serum was reacted with viral antigens prepared from BIV R29-1203 (lane 1), BIV FL491 (lane 2), an isolate of FL491 from calf 1268 (lane 3), BIV FL112 (lane 4), and an isolate of FL112 from calf 1275 (lane 5). Molecular weight markers (MWM) are indicated on the left in thousands. (B) Western blot assays of serum samples from calf 1268, which had been inoculated with BIV FL491, taken preinoculation (lane 1), on day 35 p.i. (lane 2), and on day 105 p.i. (lane 3) and serum samples from calf 1275, which had been inoculated with BIV FL112, taken preinoculation (lane 4), on day 35 p.i. (lane 5), and on day 91 p.i. (lane 6). Molecular weight markers are shown on the left. The 20-kDa band in pre- and postinoculation serum samples from both calves is not BIV specific.

increased, with an increased percentage of mononuclear cells, but remained within the normal range (Fig. 3). The calves had the expected prescapular lymph node enlargement on the side on which the inoculation materials were injected but showed no other clinical symptoms.

Preinoculation sera from all calves were negative for neutralizing antibodies to BVDV, BLV, and BSV, except for calf 848, which had neutralizing antibody to BVDV. After inoculation with BIV, the three BVDV-seronegative calves remained negative for virus-neutralizing antibodies to

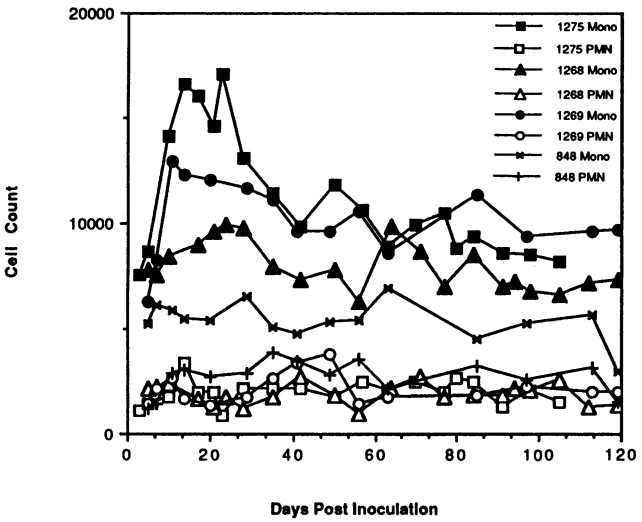

FIG. 3. Comparison of mononuclear (Mono) and polymorphonuclear (PMN) cell counts in two calves inoculated with BIV FL112 (calves 1275 and 1269) or BIV FL491 (calves 1268 and 848). Calves 1275 and 1269 showed the greatest mononuclear cell increase, similar to the WBC increase reported in the original experimental inoculations with BIV R29 (31). No changes in the polymorphonuclear cell population were observed for either calf.

BVDV. AGID tests for antibodies to BLV and BSV also remained negative for multiple samples taken from all calves from weeks 7 through 14 p.i.

The new isolates in experimental inoculations caused a leukocytosis similar to that described in the original experimental inoculations with BIV (28). More cattle need to be inoculated to confirm the initial finding that these new isolates cause leukocytosis, to rule out the possibility of biological variation among cattle. These studies may provide further insight into the question of possible attenuation of the BIV R29 isolate and verify that BIV does cause leukocytosis. Results from the current study, as well as from future studies performed with these new wild-type isolates, should provide a clearer picture of the true effect of experimental inoculation with BIV.

In conclusion, this study provides information on the biological and genetic characteristics of BIV and provides new BIV isolates for experimental study. Since it is possible that the original BIV R29 isolate was attenuated through multiple cell culture passages, these new, low-passage, contaminant-free isolates will allow measurements of possible immune suppression in vivo that may more accurately reflect a natural infection. The new isolates of BIV are important for continued investigation of the pathogenesis of BIV infections and elucidation of the potential role of BIV as a disease agent or a cofactor in disease. The new isolates increase the potential value of BIV as an animal model for lentivirus infections.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences reported here are L06524 (BIV FL112), L06525 (BIV FL491), and L06526 (BIV R29-1203).

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