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# Examination of whether Persistently Indeterminate Human Immunodeficiency Virus Type 1 Western Immunoblot Reactions Are Due to Serological Reactivity with Bovine Immunodeficiency-Like Virus

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**The bovine lentivirus, known as bovine immunodeficiency-like virus (BIV), is genetically, structurally, and antigenically related to human immunodeficiency virus type 1 (HIV-1). It is not known whether sera from persons exposed to BIV proteins would show either positive or indeterminate reactivity on HIV-1 antibody tests. We used a BIV Western blot (immunoblot) analysis to examine human sera characterized as HIV-1 antibody positive, HIV-1 antibody negative, HIV-1 persistently indeterminate, HIV-1 p17 antibody positive only, HIV-1 p24 antibody positive only, human T-cell leukemia virus type 1 (HTLV-1) p19 antibody positive only, or HTLV-1 p24 antibody positive only. None of these sera were positive by Western blot to BIV-specific proteins. Many of these sera, however, displayed strong reactivities to bovine cell culture antigens on blots prepared from both mock-infected and BIV-infected cell cultures. The HIV-1 p17 and p24 antibody-positive and the HTLV-1 p19 and p24 antibody-positive sera were further examined by Western blot to bovine leukemia virus (BLV) and were found to be negative. We examined sera from laboratory personnel at risk for BIV exposure, including two laboratory workers who were exposed to BIV by accidental injection with BIV-infected cell culture material, and found no evidence of seroconversion to BIV-specific proteins. We tested 371 samples of fetal bovine sera, each sample representing serum pooled from one to three fetuses. All samples were negative by BIV Western blot. To date, we have not detected any human sera with antibody to BIV-specific proteins. Our data indicate that persistently indeterminate results on HIV-1 Western blot are not caused by a human antibody response to BIV proteins.**

Bovine lentivirus (*Lentivirinae*), previously known as bovine visna-like virus (5) and bovine visna virus (14), was originally isolated from peripheral blood leukocytes and lymph nodes of cattle suspected of having lymphosarcoma (41). Because of structural, genetic, antigenic, and biological similarities to human immunodeficiency virus type 1 (HIV-1) (6, 15–17), the bovine lentivirus has been designated bovine immunodeficiency-like virus (BIV). BIV is morphologically and antigenically distinct from two other bovine retroviruses, bovine leukemia virus (BLV) (*Oncovirinae*) and bovine syncytial virus (*Spumavirinae*) (43).

The HIV-1 Western blot (immunoblot) assay has been widely used as the confirmatory laboratory procedure to establish whether or not a positive anti-HIV-1 enzyme immunoassay screening test represents true exposure to HIV-1. There are instances when HIV-1 Western blot patterns are neither reactive nor nonreactive but have indeterminate patterns, characterized by reactivities with viral core proteins, and do not show reactivities sufficient to be interpreted as diagnostic of HIV-1 infection. The occurrence of indeterminate Western blot patterns in HIV-1-seronegative individuals is well documented (7, 8, 21, 23, 26, 35). In the United States, the frequency of persons from low-risk groups who test indeterminate on HIV-1 Western blot is 15 to 20% (35). In these low-risk groups, many studies have shown that the persistently indeterminate Western blot pattern is usually not due to infection with HIV-1 (7, 8, 23, 26). The cause(s) of these reactions, however, is still unknown. Several authors have suggested that exposure to other, yet unknown retroviruses (4, 12) or, more specifically, exposure to bovine retroviruses may be a reason for indeterminate HIV-1 serological reactions (4, 7, 20). The similarities between HIV-1 and BIV are a concern because humans consume beef and dairy products, and fetal bovine serum is a component of most vaccines prepared in cell cultures. Both of these situations create a potential for human exposure to bovine retroviruses and bovine retrovirus proteins. It is not known whether exposure to BIV or BIV proteins can cause human sera to display either false-positive or persistently indeterminate antibody reactions on HIV-1 tests. It has also not been determined whether humans develop antibodies to BIV proteins. Until recently (45, 46), there was no serological assay for BIV that could be used to answer these questions.

There has been no report on whether human positive, false-positive, or persistently indeterminate HIV-1 sera are reactive on Western blots to proteins of BLV. Sequence data show that BLV and the human T-cell leukemia virus type 1 (HTLV-1) are phylogenetically closely related type C retroviruses (34). There has been one report (28) of cross-reactive antibodies to the *gag* protein between BLV and HTLV-1. Although BLV and HIV-1, a lentivirus, are not closely related phylogenetically, one study (40) showed that rabbit

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antisera to proteins of BLV react with HIV-1-infected lymphocytes.

The objectives of the study reported here were (i) to examine whether persistently indeterminate HIV-1 Western blot patterns of human sera are caused by antibody responses to bovine retrovirus proteins, especially the bovine lentivirus BIV; (ii) to examine whether fetal bovine serum, a common supplement of cell culture medium, contains antibodies to BIV, and thus could be a potential source of exposure to BIV proteins; and (iii) to examine whether humans at risk for exposure to BIV develop antibodies to that virus.

## MATERIALS AND METHODS

**Viruses and cell cultures.** The R29 isolate of BIV (41) was propagated on fetal bovine lung cell cultures by a cocultivation method that has been described previously (46). BLV was obtained from the FLK-BLV cell line (29).

**Serum specimens.** A panel of 24 human serum samples from healthy blood donors was previously characterized by the HIV-1 Western blot as positive (4 serum samples), negative (4 serum samples), or persistently indeterminate (16 serum samples) (see Table 1). These sera were tested on both a BIV Western blot and a bovine uninfected cell control Western blot. All of the persistently indeterminate sera had exhibited that pattern for 6 months to 3 years. A separate panel of 58 human serum samples that were characterized by Western blot as persistently indeterminate to HIV-1 p24 only (15 serum samples), HIV-1 p17 only (10 serum samples), HTLV-1 p24 only (21 serum samples), or HTLV-1 p19 only (12 serum samples) were tested by using BIV and BLV Western blots. Control BIV sera included bovine anti-BIV hyperimmune sera (46) and bovine anti-BIV serum from an experimentally infected animal.

Human serum samples from eight laboratory workers and three animal care technicians who work with concentrated virus and/or BIV-infected animals were examined by the BIV Western blot. Sera included preexposure samples, taken before work with BIV commenced, and postexposure samples, taken at regular 6- to 12-month intervals thereafter. Also included in this group of samples from at-risk individuals were pre- and postexposure sera from two laboratory personnel who were exposed to BIV-infected cell culture material after accidental needle stick injections while inoculating animals.

In order to test the serological cross-reactivities of HIV-1 and BIV with some of the common bovine retroviruses as well as ovine, caprine, and equine lentiviruses, reference sera were tested by both BIV and HIV-1 Western blots. These sera included anti-BIV hyperimmune bovine serum; anti-BIV serum from an experimentally infected ovine; anti-BSV serum from an experimentally infected bovine; anti-BLV serum from a naturally infected bovine; anti-BLV serum from an experimentally infected ovine; anticaprine arthritis-encephalitis virus (CAEV) serum from an experimentally infected caprine and antiovine progressive pneumonia virus serum from an experimentally infected ovine supplied by M. J. Schmerr, National Animal Disease Center, Ames, Iowa; antiequine infectious anemia virus (EIAV) serum pool from naturally infected equines supplied by the National Veterinary Services Laboratories, Ames, Iowa; human anti-HIV-1 Western blot reference serum from Ortho Diagnostic Systems, Raritan, N.J.; and rabbit anti-HIV-1 p24 serum, rabbit anti-HIV-1 gp160/41 serum, rabbit anti-HIV-1 p17 internal peptide-specific serum, and rabbit anti-HIV-1 p17 COOH-terminus peptide-specific serum supplied by Cambridge Biotech Corp., Rockville, Md.

Three hundred seventy-one samples of fetal bovine serum, which were supplied by S. Becvar, National Animal Disease Center, Ames, Iowa, were tested for antibodies to BIV by Western blot. Each sample represented serum pooled from one to three bovine fetuses. The samples were obtained from a supplier who collected them at abattoirs in South Dakota, Minnesota, and Illinois.

**Western blot analyses.** Antigens for the BIV, BLV, and uninfected cell culture control Western blots were prepared by differential centrifugation as described previously (46). Western blots were performed by a method described previously (45). Briefly, proteins were separated by gradient sodium dodecyl sulfate–10 to 20% polyacrylamide gel electrophoresis on preformed minigels (Enprotech, Hyde Park, Md.), electroblotted onto nitrocellulose, and blocked with 0.05% Tween 20 in TS buffer (10 mM Tris, 150 mM NaCl [pH 8.6]). Primary test sera were diluted 1:10 in TS and incubated on the blots for 2 h at room temperature in a 28-well miniblot apparatus (Immunetics, Cambridge, Mass.). Blots were rinsed twice in TS, removed from the miniblot apparatus, rinsed for 15 min in TBS (20 mM Tris, 500 mM NaCl [pH 7.5]), and then incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated recombinant protein G in TBS. Blots were rinsed three times in TBS and were then developed by using a 4-CN membrane peroxidase substrate kit (Kirkegaard & Perry, Gaithersburg, Md.).

The HIV-1 Western blot strips used were components from U.S. Food and Drug Administration-licensed Cambridge Biotech HIV-1 Western blot kits and were provided by Ortho Diagnostic Systems. Sera were tested on the HIV-1 strips according to the protocol of the manufacturer, except that horseradish peroxidase-conjugated protein G instead of antihuman conjugate was used.

## RESULTS

**Human sera.** Virus-specific antibody profiles on HIV-1 and BIV Western blot strips are presented in Fig. 1. The reactions of bovine anti-BIV and human anti-HIV-1 sera on BIV and HIV-1 antigen strips, respectively, show that although both viruses have similar polypeptide patterns, the proteins are not identical in size. Polypeptide designations for BIV have been assigned previously (15, 45, 46) and putatively designated on the basis of immunoreactions with proteins predicted by the nucleotide sequence (13).

When the panel of 24 human serum samples (Table 1) that were previously characterized as HIV-1 Western blot positive, negative, or persistently indeterminate were tested by using both BIV Western blot and bovine uninfected cell control Western blot, all of the sera were negative for antibodies to BIV. Many sera, however, displayed strong reactivities to both mock-infected and BIV-infected bovine cell culture antigens. The Western blots of 20 representative serum samples are presented in Fig. 2. Bands that were either not present (lanes 4, 11, 19, and 21) or weakly present (lanes 4 and 11) in the mock-infected cell lysate control appeared. None of these bands directly correlated with BIV-specific proteins compared with the bands in lanes C1 and C2. Although the people from whom test sera were obtained had a wide range of exposures to animals or consumed unpasteurized milk, as determined by a questionnaire (Table 1), there was no apparent correlation between these exposures and the strong reactivities to bovine cell culture proteins.

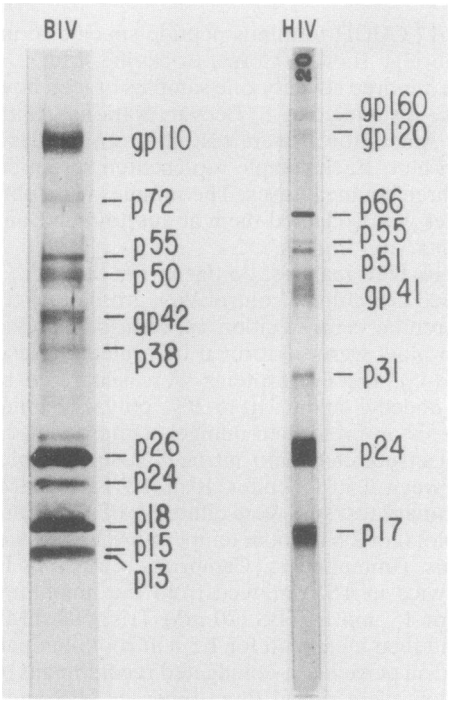


FIG. 1. Typical positive Western blot patterns for BIV and HIV-1. The BIV antigen strip was reacted with bovine anti-BIV serum, and the HIV-1 antigen strip was reacted with human anti-HIV-1 serum. Major viral polypeptides are indicated on the right of each strip by their relative molecular sizes.

TABLE 1. Identification and history of human serum samples[a] that were characterized by HIV-1 Western blot as positive, negative, or persistently indeterminate

| Serum sample | HIV Western blot reactivity | Animal exposure[b] | Ingestion of unpasteurized milk |
|---|---|---|---|
| 1–3 | Positive | Household | No |
| 4 | Positive | Household | Yes |
| 5 | Negative | Household, farm | Yes |
| 6 | Negative | Dairy, hunting | Yes |
| 7 | Negative | Household, dairy | Yes |
| 8 | Negative | Household, dairy, hunting | No |
| 9 | p24 | Household, dairy | Yes |
| 14 | p24 | Household | No |
| 15 | p24 | Household, dairy, hunting | Yes |
| 17 | p24 | Household, dairy | Yes |
| 10 | p17 | Household, hunting | Yes |
| 18 | p17 | Household, dairy | Yes |
| 19 | p17 | Household, dairy, hunting | Yes |
| 11 | p17, occasional p55 | Household | Yes |
| 12 | p24, p51, p66 (once) | Household | No |
| 13 | p17, p24, p31, p51, p55 (twice, weak) | Household | Yes |
| 16 | p24, p55 | None | No |
| 20 | p17, p24 | Household | Yes |
| 23 | p17, p24 | Household, dairy | Yes |
| 21 | p17, p24, p51 | Household | No |
| 22 | p24, p51, p55 | Household, hunting | Yes |
| 24 | p24, gp41 (twice) | Household | Yes |

[a] Serum samples were from the American Red Cross Blood Services, Syracuse, N.Y.

[b] Animal exposures were as follows: household, dogs, cats, birds, fish, gerbels, guinea pigs; dairy, cows; farm, sheep, horses, pigs; hunting, deer, bear, rabbits. Exposure was determined by answers to a questionnaire.

The second panel of 58 human serum samples that were Western blot positive either to p24 or p17 of HIV-1 or to p24 or p19 of HTLV-1 were all negative for antibodies to either BIV or BLV proteins, as determined by Western blot assays (data not shown).

Sera from humans considered to be at risk for BIV exposure, either by working with concentrated virus or BIV-infected animals or after accidental injection with BIV-infected cell culture material, were negative for antibodies to BIV on Western blot (data not shown).

**Fetal bovine serum.** All 371 samples of fetal bovine serum, each sample representing serum pooled from one to three fetuses, were negative for BIV antibodies by Western blot (data not shown). Because bovine maternal antibodies do not cross the placenta, antibodies present in the fetus are produced by the fetus and indicate in utero fetal infection. The absence of BIV antibodies in the fetal bovine sera tested in the current study indicates that fetal bovine serum is probably not a likely source for human exposure to BIV or BIV proteins.

**Serological cross-reactivities.** Results of both BIV and HIV-1 Western blots that examined the serological cross-reactivities of HIV-1 and BIV with other bovine retroviruses as well as with ovine, caprine, and equine lentiviruses are presented in Fig. 3. Anti-BSV (Fig. 3, lanes 3) and anti-BLV (Fig. 3, lanes 4 and 5) sera did not react on the BIV blot, indicating a lack of serological cross-reactivity between BIV and the other two bovine retroviruses tested. Among the bovine retrovirus sera tested (Fig. 3, lanes 1 to 5), only the serum from a BIV hyperimmunized bovine (Fig. 3, lanes 1) showed a weak p24 response on HIV-1 Western blot. Similarly, rabbit anti-HIV-1 p24 serum (Fig. 3, lanes 10) was reactive with BIV p26. Conversely, serum samples from a BIV-infected animal (Fig. 3, lanes 2) or an HIV-1-infected human (Fig. 3, lanes 9) did not cross-react. Cross-reactivities with the hyperimmune sera were limited to p24 and p26 of HIV-1 and BIV, respectively. Rabbit antisera to HIV-1 *env* (Fig. 3, lanes 11) and HIV-1 p17 (Fig. 3, lanes 12 and 13) did not react with BIV polypeptides on Western blot. Antisera to the caprine (Fig. 3, lanes 6) and ovine (Fig. 3, lanes 7) lentiviruses did not react on either the BIV or the HIV-1 Western blot. Anti-EIAV serum (Fig. 3, lanes 8) showed serological cross-reactivities with both viruses, reacting with *gag* and *gag*-associated polypeptides of BIV and both *gag* (p24) and *env* (gp160) proteins of HIV-1.

## DISCUSSION

We previously reported (45, 46) that Western blot analysis could be used to detect BIV antibodies in experimentally and naturally infected animals. To date, using the BIV Western blot, we have not been able to detect antibody to BIV proteins in human sera. It is now generally accepted that persistently indeterminate reactions on HIV-1 Western blot are not indicative of an HIV-1 infection (7, 8, 23, 26). The cause(s) of those reactions, however, remains unresolved. Results from the study presented here indicate that human sera that display persistently indeterminate HIV-1 Western blot patterns are not immunoreactive with BIV-specific polypeptides on Western blot. All of the serum samples from a group of eight laboratory workers and three animal care

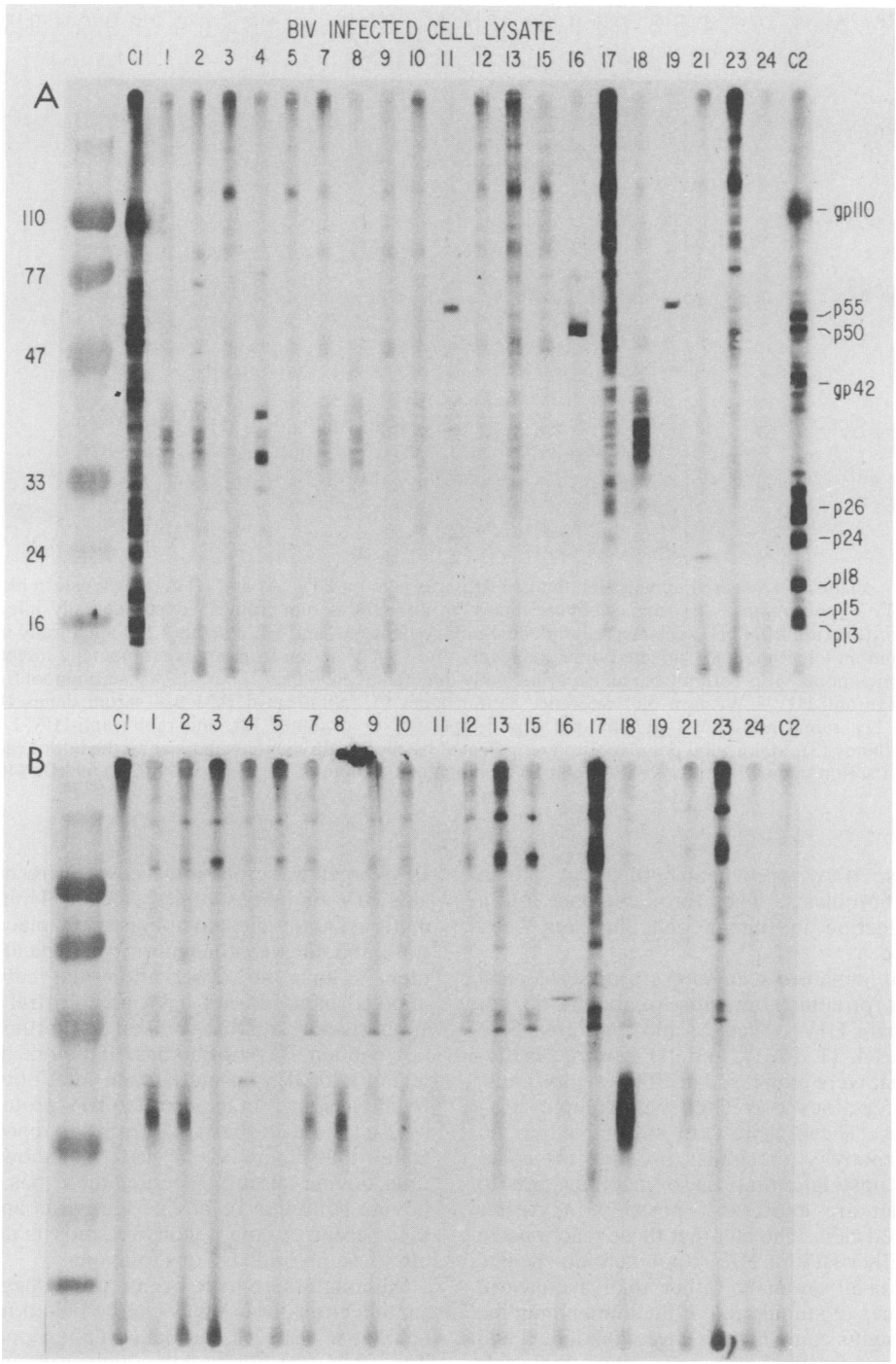


FIG. 2. BIV Western blot and bovine uninfected cell control Western blot analysis of selected human blood donor serum samples (see Table 1). (A) BIV-infected cell lysate. (B) Mock-infected cell lysate. Identification of the serum sample tested is given above each lane and corresponds to the designation given in Table 1. Sera were characterized previously, by using HIV-1 Western blot, as positive (serum samples 1 to 4), negative (serum samples 5 to 8), or persistently indeterminate (serum samples 9 to 24). Positive control BIV sera included anti-BIV hyperimmune bovine serum (lane C1) (46) and anti-BIV serum from an experimentally infected ovine (lane C2). Molecular size markers are shown on the left, and the major BIV polypeptides, identified by their relative molecular sizes, are indicated on the right.

technicians considered to be potentially at risk for exposure to BIV were negative on BIV Western blot. Two laboratory workers who were accidentally injected with BIV-infected cell culture material did not develop antibodies to BIV. This is in agreement with recent reports that BIV does not produce a productive infection in human cell cultures (17, 25). An earlier report by Georgiades et al. (14) indicated that human fibroblastoid cell cultures from leukemic bone marrow could be infected with the bovine lentivirus. However, in that report the authors noted that infectious virus could

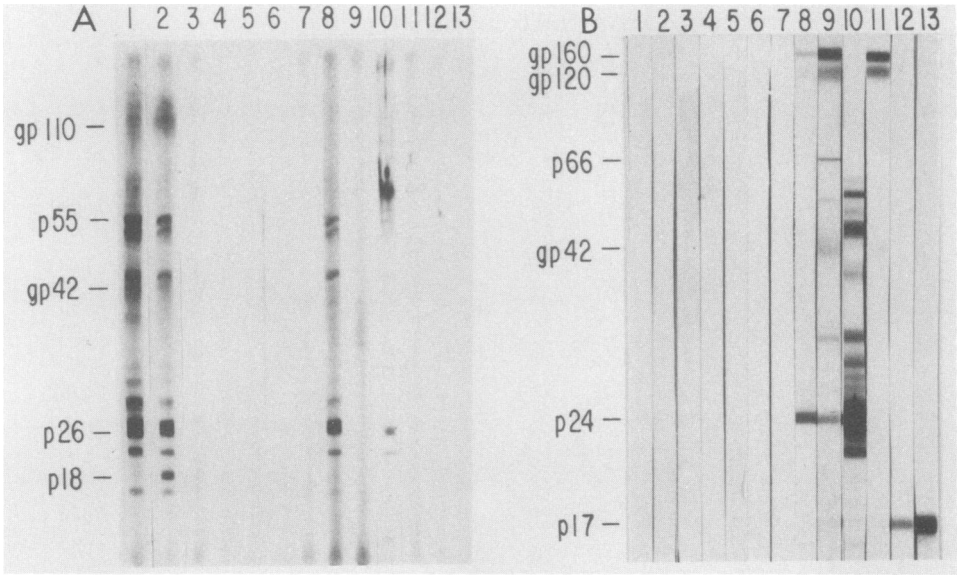


FIG. 3. Western blot analysis of selected human and animal retrovirus sera on BIV (A) and HIV-1 (B) Western blots. The sera used on each blot were anti-BIV hyperimmune sera from a bovine (lanes 1), anti-BIV serum from an experimentally infected ovine (lanes 2), anti-bovine syncytial virus serum from an experimentally infected bovine (lanes 3), anti-BLV serum from a naturally infected bovine (lanes 4), anti-BLV serum from an experimentally infected ovine (lanes 5), anti-CAEV serum from an experimentally infected caprine (lanes 6), antiovine progressive pneumonia virus serum from an experimentally infected ovine (lanes 7), anti-EIAV serum pool from naturally infected equines (lanes 8), human anti-HIV-1 Western blot reference serum (lanes 9), rabbit anti-HIV-1 p24 serum (lanes 10), rabbit anti-HIV-1 gp160/41 serum (lanes 11), rabbit anti-HIV-1 p17 internal peptide-specific serum (lanes 12), and rabbit anti-HIV-1 p17 COOH-terminus peptide-specific serum (lanes 13). Major viral polypeptides are indicated by their relative molecular size to the left of each panel. On the BIV blot, the rabbit anti-p24 serum (lane 10) reacted with a non-BIV bovine cell protein of approximately 62 to 65 kDa (46).

not be rescued. Also, they were unable to infect human diploid embryonic fibroblasts. Therefore, evidence for a productive BIV infection in human cells has not been demonstrated to date.

Our data show that humans can develop strong serological responses to bovine proteins, but those reactions do not resemble indeterminate HIV-1 Western blot patterns. Five samples (Fig. 2, lanes 4, 11, 16, 19, and 21) showed reactivities with proteins that were expressed in BIV-infected cells, but none of those reactions correlated with known BIV-specific polypeptides. These same sera showed either no reactivity or low reactivity to similar proteins in mock-infected cells. We postulate that these reactions are to cellular proteins that are expressed, possibly in greater abundance, in infected cells. The fact that these reactions do not correlate with either BIV or HIV-1 polypeptide-specific reactions make them an anomaly rather than a concern. Some potential sources of stimulation of the human immune system to bovine proteins could include ingestion of beef and dairy products and vaccination with cell culture materials that contain fetal bovine serum. Among the serum samples tested in this study, there was no apparent correlation between animal exposure or consumption of unpasteurized milk and the production of antibodies to bovine cell culture antigens.

None of the fetal bovine sera that we examined contained antibodies to BIV. The abattoirs where serum samples were collected were located in South Dakota, Minnesota, and Illinois. Although this does not guarantee that the cattle were from these states, it increases the likelihood that the cattle were from the central United States. There are no data available to indicate the rate of seropositivity among cattle in that region. We have observed that seven calves born to five BIV-infected, seropositive cows were negative for detectable BIV antibodies at birth (43, 44). Unlike humans, bovine maternal antibodies do not cross the placenta, and precolostral antibodies in a newborn calf indicate fetal infection in utero. Thus, our observation that calves born to BIV-infected dams do not have precolostral antibodies to BIV supports our data that the fetal calf serum samples tested did not contain BIV antibodies. If bovine fetuses are not infected with BIV in utero, then fetal bovine serum is not a potential source of exposure to BIV proteins. As pointed out by Lucas and Roberts (27), a prior report (14) erroneously stated that bovine visna virus was shown to be present in fetal bovine serum. To date, there has been no report of bovine lentivirus or bovine lentivirus antibody in fetal bovine serum. A comprehensive study is needed to substantiate these preliminary observations.

Although there have been reports suggesting that retrovirus infections of bovines can be linked to AIDS and HIV-1 serology (7, 20, 37), our data do not support this hypothesis. The BLV and bovine syncytial virus antisera used in this study were negative not only on BIV Western blots but also on HIV-1 Western blots. Although the data presented here show serological cross-recognition between HIV-1 p24 and BIV p26, this cross-reactivity was demonstrated with sera from animals that had been given repeated injections of viral proteins, and cross-reactivity was not evident with sera from a BIV-infected animal or an HIV-1-infected human. It is interesting that, although the rabbit anti-HIV-1 p24 serum was also strongly reactive with all of the HIV-1 *gag* precursor and intermediate polypeptides, it reacted very weakly with all of the BIV *gag*-associated polypeptides except the major core antigen p26. There has been a report (40) that rabbit antisera to proteins of BLV react with HIV-1-infected

lymphocytes. When we examined human sera that reacted with HIV-1 p24 or p17 only or with HTLV-1 p24 or p19 only, we saw no evidence of virus-specific reactions on either BIV or BLV Western blots. Our data suggest that the limited serological cross-reactions between human and bovine retroviruses that have been reported (28, 40) as well as observed in the study described here are probably indicative of immune system recognition of highly conserved peptides (epitopes) among these phylogenetically related retroviruses and that these cross-reactions are more readily detected when serum samples from hyperimmunized animals are used.

Serological cross-reactivities among many of the lentiviruses have been reported (4, 10, 11, 15, 18, 32, 33, 36). Cross-reactivities of heterologous lentivirus antisera with BIV antigens on Western blot have not been reported. In the current study, we were unable to demonstrate reactions on either BIV or HIV-1 Western blots using the ovine or caprine antilentivirus sera. Similar results with HIV-1 were reported by Goudsmit et al. (18) by using ovine anti-visna virus and caprine anti-CAEV sera. Gonda et al. (15) showed cross-reactivity with CAEV by using rabbit anti-HIV-1 serum, but they were unable to show cross-reactivity by using rabbit anti-BIV serum. In the same study, cross-reactivity with visna virus was undetected with either the HIV-1 or BIV rabbit antiserum. Investigators have previously demonstrated a one-way cross-reactivity of EIAV-positive serum with HIV-1 *env* (32, 36) and *gag* proteins (31). Nucleic acid hybridization and sequence analyses have shown relatedness between EIAV and HIV-1 *gag-pol* sequences (33, 39). Gonda et al. (15) showed that rabbit antiserum to HIV-1 is weakly reactive to EIAV on Western blot, whereas rabbit antiserum to BIV did not react with EIAV polypeptides. In the current study, using a pool of EIAV-positive equine sera derived from infected horses, we showed strong Western blot reactions with *gag* and *gag*-associated polypeptides of BIV and both the *gag* (p24) and the *env* (gp160) proteins of HIV-1.

Our results show that HIV-1 persistently indeterminate Western blot serology is not caused by immune system recognition of BIV or BLV proteins. There has been public concern (1–3, 7, 11, 19, 20, 22, 24, 37, 38) over the similarities between BIV and HIV-1 and BLV and HTLV-1. Past studies (9, 30, 42) indicate that BLV does not present a zoonotic capacity. Data from our study fail to show any evidence that BIV has a zoonotic potential.

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