High Prevalence of Borna Disease Virus Infection in Healthy Sheep in Japan

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Received 4 November 1996/Returned for modification 6 January 1997/Accepted 4 March 1997

Borna disease virus (BDV) is a neurotropic, as-yet-unclassified, negative-sense, single-stranded RNA virus (12, 35). BDV naturally infects horses and sheep and induces a disease characterized by progressive meningoencephalopathy (17, 24, 25, 30, 38). In addition, BDV naturally infects cats, cattle, and ostriches. BDV-specific antibodies are readily detected in these animals even when there are no clinical signs, suggesting that BDV is more widespread than previously thought (16, 19, 23, 27, 28). BDV may be closely associated with specific psychiatric disorders in humans, since serum antibodies to BDV have been detected at high rates (1, 4, 6–9, 15, 31–33, 37, 39). Similarly, even healthy individuals or patients without psychiatric disorders may contain antibodies to BDV, although the prevalence is very low (6–8, 31).

We developed a molecular epidemiological protocol (21, 22, 27–29) to detect BDV RNA in peripheral blood mononuclear cells (PBMCs) from healthy sheep bred on the same island. The BDV prevalences by immunoblotting and/or reverse transcriptase PCR were 0% (0 of 19) in newborns (<1 month old), 51.7% (15 of 29) in lambs (1 to 6 months old), and 36.7% (11 of 30) in adults (>2 years old). Among animals positive for BDV, 60% of lambs and 45.5% of adults contained BDV RNA in PBMCs while 46.7% of lambs and 90.9% of adults contained specific antibodies to BDV. Thus, it is suggested that virus replication in the blood, as observed in lambs, is usually reduced in adulthood by raising immune responses to BDV.

Materials and Methods

Plasma and PBMC preparations from animals. We examined BDV seroprevalences in sera stored at −70°C and prepared from 317 healthy adult sheep at a Hokkaido farm in 1989. In addition, EDTA-treated blood samples were also obtained from 19 (1 through 19) newborns (<1 month old; mean age, 20.3 days; 29 (20 through 48) lambs (1 to 6 months old; mean age, 3.2 months), and 30 (49 through 78) adults (2 to 7 years old; mean age, 4.8 years), all of which were reared on the same farm during 1995 and 1996. No original sheep from 1989 was included in the adult group of 30 sheep tested in 1995 and 1996. None of these animals showed neurological symptoms. After plasma was removed from EDTA-treated blood samples, PBMCs were isolated by Ficoll-Conray centrifugation (density, 1.087 g/ml).

Virus and cells. The controls were uninfected MDCK cells or MDCK cells persistently infected with a horse-derived BDV (MDCK/BDV) (18).

Detection of anti-BDV antibodies. Anti-BDV antibodies in serum and plasma samples were detected by enzyme-linked immunosorbent assay (ELISA) or immunofluorescence with peroxidase-conjugated rabbit immunoglobulin G (IgG) fraction to sheep immunoglobulin G (Organon Teknika Corporation, Durham, N.C.), as previously described (2, 22). The BDV antigens for these assays are ORF 1 and ORF II, coding for p40 nuclear protein and p24 polymerase cofactor, respectively (12, 35). Horse BDV-derived recombinant full-length p40 and p24 fusion proteins with glutathione S-transferase (GST) were expressed in Escherichia coli, as previously described (3, 22). The negative control antigen was GST alone. These GST-p40, GST-p24, and GST proteins were purified by glutathione-Sepharose 4B (Pharmacia Biotech AB) column chromatography before being used.

Extraction of total cellular RNA. Total cellular RNA was prepared by using an RNA extraction kit (RNAzol B; Cinna/Biotex Laboratories International, Inc.), as previously described (10).

RT-PCR. Extracted RNA was amplified by nested RT-PCR, as previously described (22), to obtain a fragment of the p24 coding region which is relatively conserved within the BDV genome (36). The control experiment included uninfected MDCK cells or persistently infected MDCK/BDV cells. Briefly, 1 μg of cellular RNA was amplified by nested RT-PCR with two sets of primers as follows: for the first PCR, nucleotides 1387 through 1405 and 1865 through 1847, and for the second PCR, nucleotides 1443 through 1461 and 1834 through 1816. RT-PCR, consisting of reverse transcription and amplification of viral cDNA, was performed according to the protocol supplied with the EZ•Telh RNA PCR kit (Perkin-Elmer Corporation). PCR products were separated by 1.5% agarose gel electrophoresis, blotted onto a nylon membrane, and Southern hybridized with four 32P-labeled synthetic oligonucleotides, sense nucleotides 1462 through 1485, 1485 through 1507, and 1637 through 1658 and antisense nucleotides 1811 through 1791.

Cloning with subsequent sequencing of PCR products. PCR products were cloned into a pUC18 plasmid vector (Pharmacia Biotech AB). Several representative clones from individual sheep were sequenced according to the protocol of
the dye primer cycle sequencing kit (Applied Biosystems) by using the 21M13 dye primer and M13 reverse dye primer in a 373-A DNA sequencer. The nucleotide sequences were analyzed with GENETYX-MAC (Software Development Co., Ltd., Tokyo, Japan). All the BDV nucleotide sequence numbers described here correspond to the previously reported numbering scheme for strain V of BDV (11, 36).

Nucleotide sequence accession numbers. The BDV p24 sequences are available in the DDBJ, EMBL, and GenBank DNA databases under accession no. AB001470 for clone 49-1, AB001471 for clone 49-5, AB001472 for clone 72-2, AB001473 for clone 72-4, AB001474 for clone 59-1, and AB001475 for clone 59-2.

RESULTS

Varied BDV prevalences in newborns, lambs, and adults. Initially, the sera of 317 adult sheep obtained from one farm on the island of Hokkaido in 1989 were examined by ELISA to determine the presence of anti-BDV antibodies. GST-p24 and GST alone (as a control) were purified by affinity column chromatography. The results were evaluated after the absorbance (at 492 nm) values for GST alone had been subtracted from those of GST-p24 in individual samples (Fig. 1A). The mean absorbance ± standard deviation was 0.5 ± 0.3. Sera from 85 of 317 (26.8%) sheep had an absorbance of >0.6. Immunoblotting of randomly selected sera with GST-p24 and GST proteins confirmed the specific reactions of four sera with an absorption (at 492 nm) of at least 0.6 by ELISA (Fig. 1B). Similar positive reactions to GST-p40 were also detected in these sera. Thus, the seroprevalence in sheep was quite high.

Based on these results, we collected EDTA-treated blood samples from sheep at the same farm during 1995 and 1996. These sheep included 19 newborns, 29 lambs, and 30 adults. The plasma samples were immunoblotted to identify anti-BDV antibodies. PBMC fractions from Ficoll-Conray centrifugation were analyzed by nested RT-PCR.

Plasma immunoblots showed positive signals reactive only with GST-p24, not GST, for 0% (0 of 19) of newborns, 24.1% (7 of 29) of lambs, and 33.3% (10 of 30) of adults. Profiles of representative (four positive and two negative) samples are shown in Fig. 2. The four samples positive for anti-p24 were also positive for anti-p40 antibodies. On the other hand, the results of the nested RT-PCR, which detected BDV-related RNA at the p24 region in PBMCs from these animals, were...
quite different. Signals were positive in 0% (0 of 19) of newborns, 31.0% (9 of 29) of lambs, and 16.7% (5 of 30) of adults by both ethidium bromide staining and Southern blot hybridization. Figure 3 shows representative results. All PCR products, except that from adult sheep 59, which showed a smaller (~270-bp) band, contained a discrete band corresponding in size (392 bp) to the fragment from persistent BDV in MDCK/BDV cells.

As summarized in Fig. 4, comparisons between the prevalences of lambs and adults for BDV RNA and anti-BDV antibodies revealed a significant difference. Animals positive for BDV RNA in their PBMCs were not always positive for anti-BDV antibodies in their plasma samples. The percentage positive for both anti-BDV and BDV RNA was 3.4% (1 of 29) of lambs and 13.3% (4 of 30) of adults. Consequently, the BDV prevalences by both techniques were 51.7% (15 of 29) in lambs, 36.7% (11 of 30) in adults, and 44.1% (26 of 59) in both adults and lambs, whereas it was 0% (0 of 19) in newborns. Thus, among animals positive for BDV by immunoblotting and/or RT-PCR, 60% (9 of 15) of lambs and 45.5% (5 of 11) of adults contained BDV RNA in their PBMCs while 46.7% (7 of 15) of lambs and 90.9% (10 of 11) of adults contained specific antibodies to BDV.

Among the subjects examined here, there were three cases positive for BDV between mothers and offspring i.e., lambs 21 and 22 (negative for RNA but positive for antibodies), 31 (positive for both RNA and antibodies), and 48 (negative for RNA but positive for antibodies) were born to mothers 53 (positive for RNA but negative for antibodies) and 62 and 78 (negative for RNA but positive for antibodies), respectively. In contrast, newborns 10 and 11, 12 and 13, 15 and 16, and 19 were born to mothers 72 (positive for both RNA and antibodies), 73, 75, and 77 (negative for RNA but positive for antibodies), respectively.

Comparison of p24 sequences in PCR products. Three PCR-positive animals, adults 49, 59, and 72, were randomly selected, and p24 sequences were compared. PCR fragments were cloned into the pUC18 plasmid vector. Figure 5 shows the sequences of two representative clones from each of these three individuals. The cDNA clones from adult 59 were derived from the smaller PCR product (Fig. 3). The reported p24 sequences from horse-derived BDVs in Europe, strains V,
He/80, and WT-1 (11, 36), were used as standards. The p24 sequences of two clones derived from one individual were very similar. The nucleotide sequences were also similar between BDVs derived from adults 49 and 59, which were also related to the sequence of horse-derived strain V. The nucleotide sequence from adult 72 was related to that of He/80.

The deletion in the p24 sequence of adult 59 could be due to the repeat sequence (AAG) found in the deletion junction (Fig. 5), which is also found in PCR products from psychiatric patients (22) and dairy cattle (16).

Demonstration of BDV RNA in the brains of sheep positive for serum antibodies to BDV but negative for BDV RNA in PBMCs. Among animals positive for BDV by immunoblotting and/or RT-PCR, 90.9% (10 of 11) of adults, in contrast to 46.7% (7 of 15) of lambs, contained specific antibodies to BDV (Fig. 4). Most of them were negative for BDV RNA in PBMCs. Therefore, we searched for the BDV reservoir in lamb 44, which was positive only for anti-BDV. The negative control was lamb 45, which was negative for both anti-BDV and BDV RNA.

Total RNA samples extracted from the cerebrum cortex, cerebrum (white matter), pons, medulla oblongata, hippocampus, lateral ventricle, olfactory bulb, and cerebellum of the brain were examined by nested RT-PCR under the conditions described in the legend to Fig. 3. RNA samples from the spinal cord, lymph node, liver, kidney, and spleen as well as PBMCs were also similarly characterized for BDV RNA. RT-PCR revealed BDV RNA only in the olfactory bulb of the brain from lamb 44, not in other regions of the brain, internal organs, spinal cord, or PBMCs (data not shown). BDV RNA signals were undetectable in any of these organs from lamb 45 (data not shown).

**DISCUSSION**

In this study, we focused on the BDV prevalence in healthy sheep bred on the island of Hokkaido in Japan, since we had found that horses, cattle, and cats on this island were infected with BDV at considerably high rates (16, 27, 28). By immunoblotting and nested RT-PCR, we found that the prevalence in sheep was higher than those in the animals listed above (Fig. 4). The susceptibility of sheep to BDV infection was previously reported (30, 38). In fact, it was also reported that one sheep exhibiting signs typical of Borna disease was positive for anti-BDV antibodies and BDV RNA in the cerebrum (5).

Vertical transmission from infected mothers may explain the spread of BDV. However, none of 19 newborns were positive for either BDV RNA or anti-BDV. At least seven of the newborns were born to BDV-positive mothers. Thus, these results do not support the notion of vertical transmission of BDV in sheep. However, we detected BDV-positive signals in four lambs born to BDV-positive mothers. The findings for human infants born to mothers infected with human immunodeficiency virus (HIV) indicate that this virus is transmitted in this manner. Blood HIV titers consistent with primary viremia are increased in most infants born to HIV-infected mothers at several months of age (13, 14, 26). Therefore, the presence of HIV cannot usually be diagnosed at birth, suggesting that viral transmission occurs during late pregnancy and/or delivery. Similarly, we did not detect BDV RNA in the PBMCs of any of the newborns examined. In addition, the prevalences identified by both techniques varied for different generations of sheep, with a much higher prevalence in terms of BDV RNA than of serum antibodies in lambs (60.0 versus 46.7%) but the reverse in adults (45.4 versus 90.9%) (Fig. 4). Similarly, BDV RNA was not always detected in PBMCs even from anti-BDV antibody-positive individuals, such as horses (3, 28), cattle (16), cats (27), and humans (21, 22, 29, 34). Thus, most of the BDV-positive lambs examined carried BDV RNA in PBMCs without BDV-specific antibodies, which might correspond to primary viremia. Thereafter, BDV RNA in the blood would be easily eliminated by the immune response to BDV, since we detected a high rate of specific-antibody-positive, BDV RNA-negative adults. Of particular note is the detection of BDV RNA only in the brain of an adult sheep positive for anti-BDV but negative for BDV RNA in PBMCs (data not shown). Therefore, it is likely that some animals become persistently infected with BDV in the brain even after the virus is cleared from blood cells. Thus, possible vertical transmission of BDV...
in sheep remains to be determined by extensive follow-up
studies, including cellular immune response to BDV.

The association of BDV with psychiatric disorders in hu-
mans has been postulated from the high prevalence of anti-
BDV antibodies in these patients compared with that of healthy
people (6–8, 31, 33). The demonstration of BDV RNA in PBMCs
of healthy horses (28) and cats (27), as well as of patients with
psychiatric disorders (22), patients with chronic fatigue syn-
drome (29), and blood donors (21), suggests a broader route of
natural transmission as a hematopoietic-cell-related virus than
as a highly neurotropic virus. Therefore, further comparative
studies of BDVs derived from animals and humans are quite
important for clarifying how BDV is transmitted among in-
fected animals and humans. The sequencing of PCR products
derived from PBMCs of infected animals and humans seems to
be useful for understanding the route of natural transmission
of BDV in individual host species. At present, the sequencing
results for sheep (Fig. 5) suggest close relationships between
adult 49 and 59-derived PCR products and horse-derived
strain V (11, 36) and between the adult 72-derived PCR prod-
uct and He/80 (11, 36). The close relation of sheep-derived
BDV to horse-derived BDV in the p24 region was also re-
ported by Binz et al. (5). The nucleotide sequences of adult 49
and 59-derived products show high levels of similarity with
those of BDVs derived from some patients with psychiatric
disorders (20, 22) and chronic fatigue syndrome (29) and from
horses (3) in Japan. The deletion in the PCR product derived
from adult 59 (Fig. 5) occurred at the same site as those found
in products derived from psychiatric patients (22) and dairy
cattle (16). The mutations observed here may have been PCR
artifacts from the EZ rTth PCR kit, as recently described (34).
However, such mutations would be random events and would
not cause mutations at a few nucleotide positions in cDNA
clones from each animal. In fact, a comparison between this
method and another [reverse transcription with oligo(dT)
primers followed by amplification of the resulting cDNA by a
similar nested RT-PCR] with the same RNA samples from
horses showed no apparent differences between the products
(3). Thus, these results indicate that the technique would not
be a major reason for the mutations at least at the sites com-
monly observed in cDNA from each animal.

ACKNOWLEDGMENTS

We thank R. Rott, Justus-Liebig-Universität Giessen, Giessen, Ger-
many, for providing MDCK/BDV cells.

This work was partly supported by a grant-in-aid for BDV research
from the Ministry of Health and Welfare, grants-in-aid for scientific
research (A and B) from the Ministry of Education, Science, Sports,
and Culture, and a special grant-in-aid for the promotion of education
and science at Hokkaido University provided by the Ministry of Edu-

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FIG. 5. Comparison of the p24 nucleotide sequences of BDVs derived from sheep PBMCs and horses. The sequences of p24 (nucleotides 1473 to 1772) in two
cDNA clones obtained from adults 49, 59, and 72 are shown. The reported sequences of horse-derived BDVs (strains V, He/80, and WT-1) (11, 36) are also shown
as controls. Dashes indicate deleted nucleotides. Both cDNA clones from adult 59 contained a large deletion which corresponds to the smaller band in Fig. 3. Dots
indicate nucleotides identical to those of strain V.