Detection of Borna Disease Virus- Reactive Antibodies from Patients with Psychiatric Disorders and from Horses by Electrochemiluminescence Immunoassay

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The prevalence of Borna disease virus (BDV)-specific antibodies among patients with psychiatric disorders and healthy individuals has varied in several reports using several different serological assay methods. A reliable and specific method for anti-BDV antibodies needs to be developed to clarify the pathological significance of BDV infections in humans. We developed a new electrochemiluminescence immunoassay (ECLIA) for the antibody to BDV that uses two recombinant proteins of BDV, p40 and p24 (full length). Using this ECLIA, we examined 3,476 serum samples from humans with various diseases and 917 sera from blood donors in Japan for the presence of anti-BDV antibodies. By ECLIA, 26 (3.08%) of 845 schizophrenia patients and 9 (3.59%) of 251 patients with mood disorders were seropositive for BDV. Among 323 patients with other psychiatric diseases, 114 with neurological diseases, 75 with chronic fatigue syndrome, 85 human immunodeficiency virus-infected patients, 50 with autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosis and 17 with leprosy, there was no positive case except one case each with alcohol addiction, AIDS, and dementia. Although 19 (1.36%) of 1,393 patients with various ocular diseases, 10 (1.09%) of 917 blood donors, and 3 (4.55%) of 66 multitransfused patients were seropositive for BDV-specific antigen, high levels of seroprevalence in schizophrenia patients and young patients (16 to 59 years old) with mood disorders were statistically significant. The immunoreactivity of seropositive sera could be verified for specificity by blocking with soluble p40 and/or p24 recombinant protein. Anti-p24 antibody was more frequent than p40 antibody in most cases, and in some psychotic patients antibody profiles showed only p40 antibody. Although serum positive for both p40 and p24 antibodies was not found in this study, the p40 ECLIA count in schizophrenia patients was higher than that of blood donors. Furthermore, we examined 90 sera from Japanese feral horses. Antibody profiles of control human samples are similar to that of naturally BDV-infected feral horses. We concluded that BDV infection was associated in some way with psychiatric disorders.

Borna disease virus (BDV) is a noncytopathic, neurotropic, single-strand, negative-sense RNA virus that naturally infects a wide range of vertebrate species from birds and rodents to primates. BDV is experimentally transmissible to other animal species and can also cause encephalomyelitis in a wide range of experimental animals (6, 7, 17). Because BDV-induced behavioral disturbances in animals resemble some types of psychiatric disorders in humans, it is important to determine any possible role for BDV in human mental disorders. That BDV is pathogenic for humans was first suggested by antibodies in human sera that react with BDV-infected cells and subsequently with purified BDV proteins (1, 13, 24). Recently, the recovery of infectious BDV and the detection of BDV nucleic acids in human cells support the characterization of BDV as a newly identified human pathogen (8).

The epidemiological studies were carried out by serological assays, such as indirect immunofluorescence assay (IFA), immunoprecipitation, enzyme-linked immunosorbent assay (ELISA), and Western blot (WB) analyses. However, the prevalence of BDV-specific antibodies and BDV-related RNA among patients with psychiatric disorders has varied (from 3.7% by IFA to 23.3%) in several reports (4). These assay systems are sometimes problematic; for example, due to the existence of cell-specific autoantibodies, IFA has a variability of reader interpretation and a lack of sensitivity for detecting low anti-BDV titers. Although immunoprecipitation and WB analyses (10) may be more reliable and specific than IFA for
TABLE 1. BDV seroprevalence among human patients by ECLIA with recombinant p40 and p24 proteins

| Patient diagnosis                  | Mean age + SD | % Males/ % females | No. of positive sera/total no. (%)
<table>
<thead>
<tr>
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<tr>
<td>Schizophrenia</td>
<td>49.3 ± 12.3</td>
<td>49/51</td>
<td>26/845 (3.08)</td>
</tr>
<tr>
<td>Mood disorders</td>
<td>55.6 ± 14.0</td>
<td>39/61</td>
<td>9/251 (3.59)</td>
</tr>
<tr>
<td>Alcohol addiction</td>
<td>51.3 ± 6.7</td>
<td>88/12</td>
<td>1/42 (2.38)</td>
</tr>
<tr>
<td>Mental retardation</td>
<td>38.6 ± 13.4</td>
<td>60/40</td>
<td>0/25 (0)</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>62.5 ± 12.7</td>
<td>26/64</td>
<td>1/89 (1.12)</td>
</tr>
<tr>
<td>Vascular dementia</td>
<td>78.5 ± 8.5</td>
<td>49/51</td>
<td>0/46 (0)</td>
</tr>
<tr>
<td>Other psychiatric diseases</td>
<td>57.0 ± 17.2</td>
<td>48/52</td>
<td>0/164 (0)</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
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<td>2/366 (0.55)</td>
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| Neurological diseases              | 37.2 ± 16.1   | 64/36              | 0/114 (0)                        |
| Multiple sclerosis                 |               | 0/67               |                                  |
| Encephalitis                       |               | 0/133              |                                  |
| Degenerative diseases              |               | 0/14               |                                  |
| Epilepsy                           | 43.9 ± 13.7   | 56/44              | 3/214 (1.40)                     |
| Chronic fatigue syndrome           | 32.0 ± 9.8    | 60/40              | 0/75 (0)                         |
| HIV infection (including AIDS)     | 31.9 ± 13.4   | 97/2              | 1/85 (1.18)                      |
| Autoimmune diseases                | 47.2 ± 17.3   | 43/46              | 0/50 (0)                         |
| Leprosy                            | 67.2 ± 10.4   | 53/47              | 0/17 (0)                         |
| Multitransfused patients           | 50.6 ± 18.6   | 52/48              | 3/66 (4.55)                      |
| Ocular diseases                    | 50.1 ± 19.1   | 42/58              | 19/1,393 (1.36)                  |
| Blood donors                       | 35.1 ± 13.1   | 69/31              | 10/917 (1.09)                    |

* Statistical analysis data are as follows: schizophrenia versus other psychiatric diseases ($\chi^2 = 6.16; P > 0.01$ and < 0.025), schizophrenia versus blood donors ($\chi^2 = 6.67; P > 0.005$), schizophrenia versus ocular diseases ($\chi^2 = 7.82; P > 0.01$ and < 0.01), mood disorders versus other psychiatric diseases ($\chi^2 = 6.21; P > 0.025$ and < 0.01), mood disorders versus donors ($\chi^2 = 7.67; P > 0.01$ and < 0.005), mood disorders versus ocular diseases ($\chi^2 = 6.27; P > 0.025$ and < 0.01), and multitransfused patients versus donors ($\chi^2 = 3.30; P$, not significant).

These horses do not receive any medical care or vaccinations but are observed daily for general health and social behaviors by veterinarians.

**ECLIA methods.** Two kinds of BDV protein corresponding to full-length p40 and p24 were expressed as recombinant protein in *Escherichia coli*, by using the pGEX-5X-3 vector system (Pharmacia, Upplands, Sweden), which generated the fusion protein with glutathione S-transferase (GST) and recombinant p40 or p24 protein. Each fusion protein was bound on glutathione-Sepharose 4B (Pharma- cia) and then released by factor Xa (Sigma, St. Louis, Mo.), which cleaved between GST and p40 or p24 of the fusion protein. Further, to remove the contaminating protein derived from *E. coli*, each recombinant protein was purified by ion-exchange chromatography with Mono Q (Pharmacia) followed by affinity chromatography with Sepharose 4B (Pharmacia) combined with anti-anti- clostridium immunoglobulin G (IgG) obtained from a rabbit immunized with *E. coli*. The recombinant proteins (50 μg in 0.05 M borate buffer, pH 9.5) were mixed with 2 × 10^5 microbeads overnight at 37°C. After the beads were washed three times with the washing buffer (0.05 M Tris buffer, pH 8.0, including 0.15 M NaCl and 0.01% Tween 20), and the recombinant protein-coated beads were suspended in the bead buffer (0.05 M Tris, pH 8.0, including 0.01% Tween 20 and 10% normal chicken serum) at the concentration of 10^4 beads.

**RESULTS**

(i) Detection of antibodies to BDV p40 and p24 recombinant proteins in human sera. A total of 917 sera from voluntary blood donors were tested for anti-BDV p40 and p24 antibodies by ECLIA. By using the specific inhibition paradigm, 10 sera were judged seropositive (1.09%).

Twenty-six (3.08%) of 845 schizophrenia patients and 9 (3.59%) of 251 patients with mood disorders were seropositive for BDV. The seroprevalence in young (16 to 59 years old) patients with schizophrenia (17 of 631 [2.69%]) and mood disorders (8 of 136 [5.88%]) was significantly higher than that of blood donors (10 of 917 [1.09%]) and patients with ocular diseases (6 of 916 [0.66%]) (chi-square test). Among 366 patients with other psychiatric diseases, 114 with neurological diseases, 75 with chronic fatigue syndrome, 85 human immunodeficiency virus (HIV)-infected patients including those with AIDS, 50 with autoimmune diseases, and 17 with leprosy, there were three positive cases, one patient each with alcohol addiction, AIDS, and dementia. Although 19 (1.36%) of 1,393 patients with various ocular diseases and 3 (4.55%) of 66 multitransfused patients were seropositive for BDV-specific antigen, high seroprevalence in schizophrenia patients was statistically significant.

(ii) Age and sex distribution of anti-BDV antibody. From a serological survey on BDV antibody-positive schizophrenia and mood disorder patients, the positive rate did not increase...
with age. The BDV antibody positivity rate in donors also did not increase with age. There was no difference between females and males among these psychosis patients. The BDV antibody-positive rate in males (1.16%) was higher than that in females (0.65%) among blood donors.

(iii) Profiles of antibodies to BDV recombinant proteins in humans. Although p24 antibody was more frequent than p40 antibody in most cases, in some psychosis patients antibody profiles showed only p40 antibody (Table 2). ECLIA counts of p40 antibody in most patients were higher than in healthy donors, even when ECLIA counts were under the cutoff level. The immunoreactivity of seropositive sera was verified for specificity by blocking with soluble p40 and/or p24 protein.

(iv) Detection of antibodies to BDV recombinant proteins in horses. As a first step, 21 of 90 (23.3%) Japanese feral horses had an ECLIA count of more than 1,000 by using both p40 and p24 recombinant proteins. In order to enhance specificity, an inhibition test was also used in these ECLIA. Sixteen sera were determined to be BDV seropositive (17.8%) when the addition of both BDV p40 and p24 recombinant proteins inhibited more than 50% of the original value obtained (Table 3). BDV antibody profiles in these horses were similar to that of humans; two horses had only p40 and p24 antibodies but the remainder had only the p24 antibody. There was no clinical evidence of neurological or behavioral disease observed in any of the Japanese feral horses. Misakiuma horses 77 and 41 are parent and child: the mother of horse 41 (a two-day-old baby) is horse 77. Their ECLIA counts are very similar, and the evidence of neurological or behavioral disease observed in any of the Japanese feral horses. The BDV antibody positivity rate in donors also did not increase with age. There was no difference between females and males among these psychosis patients. The BDV antibody-positive rate in males (1.16%) was higher than that in females (0.65%) among blood donors.

DISCUSSION

The ECLIA method using the conjugate labeled with ruthenium(II) Tris (bipyridyl) and magnetic microbeads was developed to provide higher sensitivity, wider dynamic range, improved precision, and shorter testing time than other conventional immunoassay methods. We used this ECLIA system to detect antibodies to BDV p40 (viral nucleoprotein) and p24 (viral phosphoprotein) in sera from two species believed to have low affinity for and low titers against BDV, humans and horses. p40 and p24, expressed at high levels in the rat brain and infected cells, represent good markers with which to search for evidence of BDV infection in animal and human sera (12). The ECLIA was able to accurately identify experimentally infected rats and horses (26). In addition, the ECLIA corresponded to IFA in domestic horses that were also seropositive by a sensitive, specific WB assay. A possible association between BDV infection and major psychiatric disorders was initially proposed after finding BDV-reactive antibodies in the sera of a small, but significant, percentage of people with these disorders, as compared to controls (13). The first studies reporting that patients with psychiatric diseases, e.g., unipolar or bipolar affective disorder, showed a higher prevalence of anti-BDV antibodies (1.6%) than healthy controls (0%) were performed by using the IFA (19). Fu et al. (10) confirmed the earlier report by using a WB assay with two BDV proteins. Walltrip et al. (25) reported a significantly higher prevalence of anti-BDV antibodies in patients with schizophrenia (13.3%) than in controls (0%), by using a WB assay.

Following the identification of BDV RNA in peripheral blood mononuclear cells (PBMC) of experimentally infected...
BDV SEROPREVALENCE IN HUMANS

There are attempts to correlate BDV RNA detection with BDV seropositivity in the same individual. Sauder et al. (22) described a similar assay used in the detection of human anti-BDV antibodies. Using this assay, Sauder et al. reported a BDV seroprevalence of 9.6% among 416 neuropsychiatric patients, versus 1.4% among 203 healthy controls. The majority of these positive sera recognized only the BDV p40 antigen. The authors also reported that three of the 13 patients whose PBMC were BDV RNA positive were also BDV seropositive, whereas one patient with serum antibodies to BDV p40 was BDV RNA negative.

However, since the overall difficulty in recovery of BDV from humans carries with it some significant false-positive and false-negative technical issues, for the foreseeable future and certainly for mass screening attempts, serological assays are likely to remain the major BDV diagnostic tests.

The ECLIA identified 10 of 917 blood donors whose sera specifically recognized BDV antigens in three different areas of Japan, although at this point we are unable to independently confirm that these individuals are infected with BDV. However, if BDV infection is present in even a small percentage of blood donors, then we have to consider initiating the screening of blood products for BDV to prevent possible iatrogenic transmission. The prevalence we observed in multitransfused patients is higher than that of blood donors, although this is not statistically significant (Yates’ correction \( \chi^2 = 3.30; P > 0.05 \) and \( < 0.1 \)). Three BDV antibody-positive patients receiving large quantities of blood products (erythrocytes and platelets) for many years (more than 12 years) are suffering from severe bone marrow dysfunction. They have been diagnosed with aplastic anemia, acute myelogenous leukemia, and paroxysmal nocturnal hemoglobinuria. Additional data from a large-scale study are needed to resolve this problem.

In our ECLIA system, the profiles of BDV antibody differed between patients with psychiatric disorders and control donors. Three of 15 patients with schizophrenia and bipolar disorder showed the antibody to only the p40 protein, and the anti-p40 antibody was not demonstrated in blood donors. Although antibodies from humans that react with both antigens have not yet been observed, ECLIA counts of p40 antibody in psychiatric patients are higher than those of blood donors. BDV p40 is a major target of the CD8 T-cell-mediated immune response in Lewis rats (16). The immune response and pathogenesis of these p40-positive psychiatric patients may be different from psychiatric patients and blood donors positive only for p24 antibodies.

Seropositive patients presented a broad spectrum of mental disorders with a predominance of deficit syndrome of schizophrenia, recurrent unipolar depression, and bipolar affective disorders. Based on these data, there is no evidence for a major clinical manifestation of infection with BDV. Since genetic background plays a role in some types of BDV-associated diseases in animals, some variability in clinical disease expression is not surprising in an outbred human population (21).

We could demonstrate very low-level BDV antibody positivity among patients with HIV infection and chronic fatigue syndrome, although there are some reports of high seroprevalence among patients with these diseases. Auwanit et al. (2) reported an unusually high seroprevalence of BDV in HIV-infected patients by ELISA with GST-BDV p24 (full-length) fusion protein as the antigen, and Nakaya et al. (15) also reported that six of 25 chronic fatigue syndrome patients were positive for BDV by WB with the same GST-BDV p24 fusion protein. In our ECLIA system, some sera from HIV-infected patients have had high counts over the cutoff level at first screening, but all except one were judged negative by a specific inhibition test. These assay systems remain problematic for specificity due to contamination of E. coli components and the use of the GST-BDV p24 fusion protein, which did not release GST. A confirmation test, such as an inhibition test with a soluble antigen, is needed to rule out a nonspecific reaction.

More than 5,000 sera from psychiatric patients and patients with unclear neurological diagnoses were investigated under blind conditions in Europe and the U.S. by IFA and immunoblot assays (20). In 4 to 7% of these sera BDV-specific antibodies could be demonstrated with titers from 1:10 to 1:640, depending on the geographic region from which the patients came. The highest percentage of seropositive patients came from a region in southern Germany where Borna disease has been known to be endemic in horses and sheep. However, approximately 1% of the 1,000 control specimens also showed antibodies to BDV irrespective of their origin.

In Japan, the high prevalence (29.8%) of BDV p24 RNA in PBMC from 57 healthy horses was demonstrated, and about 60% of the BDV RNA-positive animals showed seropositivity by WB with GST-BDV p24 fusion protein (14). However, there has so far been no report of clinically apparent Borna disease in horses. In our assay system, the feral horses in Japan also showed 18% BDV seropositivity. These horses in the Miyazaki prefecture have not been subjected to veterinary care, including vaccination or medication, for a long period of time. Thus, we can rule out the possibility that these feral horses were infected by BDV contamination of a vaccine used in domestic horses. Furthermore, we did test the detection of BDV antibody in 200 domestic horses in Japan, and 112 (56%) of these horses were BDV antibody positive (p24-positive horses, 104; p40-positive horses, 1; both p24- and p40-positive horses, 7) (unpublished data). The BDV seroprevalence of domestic horses is apparently higher than that of feral horses. The difference of seroprevalence among these two groups (feral and domestic) may depend on the circumstances of close housing and origin.

It is not known whether BDV can be transmitted from infected horses, or other animals, to humans. However, the similarity in sequence between animal and human BDV isolates (9) and experimental data showing a broad species preference for this agent suggest that animal-to-human transmission is a distinct possibility.

There is a need for epidemiological and pathological studies to rigorously evaluate the contribution of BDV to human psychiatric disorders. The ECLIA is a new, highly sensitive, and specific serological screening tool based on recombinant proteins for the measurement of anti-BDV antibody to p40 and p24 for use in investigations of human and animal BDV infections. In particular, this rapid, economical technique will be
useful for the large-scale screening required of serious epide-
miology studies of BDV in humans. The ECLIA will also be
useful in evaluating the transmission of BDV by blood prod-
ucts and will prove helpful in investigating the pathogenesis of
disease states associated with BDV infection.

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