Detection by Radioligand Assay of Antibodies against Borna Disease Virus in Patients with Various Psychiatric Disorders

Hidenori Matsunaga,1* Susumu Tanaka,2 Fuyoko Sasao,3 Yoshii Nishino,4 Masatoshi Takeda,5 Keizo Tomonaga,3 Kazuyoshi Ikuta,3 and Nobuyuki Amino6

Department of Psychiatry, Osaka General Medical Center, Osaka 558-8558,1 Department of Sleep Disorders Research, Tokyo Institute of Psychiatry, Tokyo Metropolitan Organization for Medical Research, Tokyo 156-8585,2 Department of Virology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871,3 Research Institute of Bioscience, Azabu University, Kanagawa 229-8501,4 Department of Psychiatry, Osaka University Graduate School of Medicine, Osaka 565-0871,5 and Kuma Hospital, Kobe 650-0011,6 Japan

Received 15 January 2005/Returned for modification 7 February 2005/Accepted 10 March 2005

Using a radioligand assay, which preserves the natural form of the antigen, antibodies against Borna disease virus nucleoprotein and phosphoprotein were detected in 11 and 19 sera of 171 psychiatric patients, respectively. Compared with results by Western blotting, three and nine sera were concordantly positive, respectively. The four sera showing the highest levels of antibodies by radioligand assay were all negative by Western blotting; however, dilution and inhibition tests supported the positive results. Our results suggest the importance of conformational structure to detect human anti-Borna disease virus antibodies.

Since antibodies against Borna disease virus (BDV) were found in human mood disorders in 1985 (1, 17), the role of BDV in human neuropsychiatric diseases has been investigated by means of seroprevalence (2, 18), detection of viral RNA from peripheral blood (6, 10), and examination of cerebrospinal fluids (3, 8) or autopsied brains (7, 14). However, such study has not yet established the role of BDV for human diseases (13, 16). One reason might be the lack of a reliable diagnostic system, because of the difficulty of detection of human anti-BDV antibodies compared to other animals (13), and the possible existence of the virus in the central nervous system. To detect anti-BDV antibodies, indirect immunofluorescent antibody assay (2, 4), Western blotting (9, 15), enzyme-linked immunosorbent assay (5, 11), electrophoresis and immunoblot assay (19), and T-cell proliferative response (10) have been employed, and yet disagreement remains to some extent among the results (10, 13). On the other hand, detection of BDV RNA from peripheral blood has been reported to be unreliable unless contamination of cDNAs in the laboratory is strictly excluded (13). Therefore, the establishment of a sensitive and specific method to detect anti-BDV antibodies is keenly needed to clarify the significance of BDV in humans.

Radioligand assay is highly sensitive and specific because it uses conformational antigens, and its validity has been recognized for several autoantibodies in comparison with another method (12, 20). In this study we measured anti-BDV antibodies by radioligand assay, and the results were compared with those by Western blotting.

Subjects. All samples were taken from patients of the Department of Psychiatry, Osaka General Medical Center (Table 1). This research was approved by the Center’s Ethical Committee, and written informed consent was obtained from the patients. Forty-one healthy controls were included in this study. Serum from a BDV-infected rat as well as mouse monoclonal antibodies to BDV nucleoprotein (p40; BDV-N) and phosphoprotein (p23; BDV-P) were also examined. As a standard, a “normal pooled serum” was obtained by pooling sera from 20 other healthy subjects.

Methods. To make radiolabeled antigens, the open reading frames of BDV-N and -P were obtained by reverse transcription-PCR (RT-PCR) amplification using BDV-N and -P expression vector originated from viral strain H1766 preserved in Madin Darby canine kidney cells as a template. Primer pairs were as follows. BDV-N: 5'-GGGTATCCATGCGCACCCCAAAGACGCCTGGG-3' and 5'-CCGCTCCAGCTAGTTTAGACCAGTCACACC-3'; BDV-P: 5'-GGAATTCCATGCGCACAAGCGACCATCGAGCTC-3'; and 5'-CGGCTCGAGTTATGGATATG-3'. PCR was carried out using KOD-plus (TOYOBO, Osaka, Japan) as a DNA polymerase. cDNA was digested with a BamHI or an EcoRI and ligated into the pET28a (+) expression vector (Novagen, Madison, WI). [35S]Methionine-labeled protein was produced by incubating the cDNA with [35S]methionine (Amersham Biosciences, Piscataway, NJ) in T7N Quick coupled Transcription/Translation System (Promega, Madison, WI) at 30°C for 90 min. The product was applied to nickel column (Amersham Biosciences) with reaction buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% bovine serum albumin [BSA], 0.1% Tween-20, and 0.1% NaNO3, pH 7.4) to remove free [35S]methionine.

The reaction mixtures containing 1 μl of sera and each labeled BDV protein (up to 50 μl by reaction buffer; 20 kcpm) were incubated in a 96-well deep plate overnight at 4°C. A 96-well filtration plate (Millipore, Bedford, MA) had been pretreated with blocking buffer (50 mM Tris-HCl, 150 mM NaCl, 3% BSA, and 0.1% NaNO3, pH 7.4) at 4°C overnight, and the buffer was replaced with the reaction buffer before the assay. Ten microliters of 50% Protein G Sepharose 4FF
(Amersham Biosciences) was added to each well of the
filtration plate. The reaction mixtures were then transferred
to each well and incubated with Protein G for 45 min at
room temperature. The plate was washed 10 times with
washing buffer (50 mM Tris-HCl, 150 mM NaCl, and 1%
Tween-20, pH 7.4) using a Vacuum Manifold (Millipore). The
filtration plate was dried, OptiPhase SuperMix (Perkin Elmer
Life Science, Boston, MA) was added, and the quantity of
washed antibody was measured.

### TABLE 1. Number of positive results by radioligand assay and by Western blotting

<table>
<thead>
<tr>
<th>Origin of blood sample</th>
<th>Total no. (male/female)</th>
<th>Age (mean ± SD)</th>
<th>Anti-BDV-N antibody</th>
<th>Anti-BDV-P antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RLA</td>
<td>WB</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>41 (19/22)</td>
<td>45.5 ± 13.9</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Organic brain diseases</td>
<td>9 (4/5)</td>
<td>40.2 ± 12.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Psychiatric disorders</td>
<td>171 (53/118)</td>
<td>44.1 ± 15.8</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>Schizophrenia and other psychotic disorders</td>
<td>57 (18/39)</td>
<td>43.2 ± 14.1</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Mood disorders</td>
<td>80 (22/58)</td>
<td>46.0 ± 16.9</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Other psychiatric disorders</td>
<td>34 (13/21)</td>
<td>41.9 ± 15.8</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*RLA, radioligand assay; WB, Western blotting; ND, not done.

### FIG. 1. Dilution test results for anti-BDV-N (A and C) and -P (B and D) antibodies, using serum of BDV-infected rat, mouse monoclonal antibodies (A and B), and four human sera with the highest indices from psychiatric patients (C and D). Inhibition tests were performed using the same four human sera for anti-BDV-N (E) and -P (F) antibodies and a negative human serum (E).
precipitated labeled protein was counted in 1450 MicroBeta (Perkin Elmer, Wellesley, MA). All samples were measured in duplicate. The intra-assay coefficient of variation varied from 4.3 to 6.0%, while the interassay coefficient of variation ranged from 5.7 to 9.4%. The normal pooled serum was tested in every assay in quadruplicate, and the results were expressed as “anti-BDV-N index” or “anti-BDV-P index” by calculating (cpm of the sample serum)/(cpm of the normal pooled serum).

In Western blotting, recombinant full-length BDV-N and -P fused with glutathione S-transferase ([GST]-BDV-N and -P) expressed by Escherichia coli (15) were used for antigens. The original BDV strain was the same employed to make recom-

FIG. 2. Anti-BDV-N (A) and -P (B) indices in the human samples. Solid lines show the cutoff levels for each antibody.

FIG. 3. Inhibition test results for anti-BDV-P antibody by Western blotting. In each human serum, results with (+) and without (−) absorption by [GST]-BDV-P were compared. Pm and Nm indicate mouse monoclonal antibodies against BDV-P and -N, respectively.
Other psychiatric disorders

295.3 Sch, paranoid type
295.9 Sch, undifferentiated type
295.3 Sch, paranoid type
295.3 Sch, paranoid type
295.3 Sch, paranoid type
295.3 Sch, paranoid type
295.2 Sch, catatonic type

In order to confirm the positive results and decide appropriate cutoff values, an easy dilution test was performed for samples with indices above mean plus two standard deviations (SD) of healthy controls; i.e., 3, 2, and 1 SD, respectively.

Mood disorders

300.4 Dysthymic disorder
306.39 Bipolar II disorder
306.5 Bipolar I disorder
306.6 Bipolar I disorder
306.2 MDD, single episode
306.3 MDD, recurrent
306.2 MDD, single episode
306.2 MDD, single episode
306.24 MDD, single episode

Other psychiatric disorders

300.3 Obsessive-compulsive disorder
300.7 Hypochondriasis
294.8 Anorexic disorder NOS
300.01 Panic disorder without agoraphobia
300.81 Undifferentiated somatoform disorder
294.9 Cognitive disorder NOS
305.0 Alcohol abuse
300.3 Obsessive-compulsive disorder

Results and comments. In radioligand assay, the serum of BDV-infected rat, as well as mouse monoclonal antibodies, indicated relevant dilution curves at very high dilution rates (Fig. 1A and B). Four human sera with the highest indices, three of which showed positive for both BDV-N and -P and one only for BDV-N, also showed relevant dilution curves (Fig. 1C and D). In inhibition test, the same four sera showed a considerable decrease when absorbed by the corresponding antigens, while only a slight decrease was observed when absorbed by control antigen unrelated to BDV (Fig. 1E and F). The antibody indices for BDV-N and -P of the human samples are shown in Fig. 2. In order to confirm the positive results and decide appropriate cutoff values, an easy dilution test was performed for samples with indices above mean plus two standard deviations (SD) of healthy controls; i.e., 3, 2, and 1 SD, respectively. As a result, appropriate cutoff indices were found at 2.03 (mean plus 4 SD of healthy controls) for BDV-N and at 1.36 (mean plus 2 SD) for BDV-P. Eleven (6.4%) and 19 (11%) of 171 psychiatric patients showed a positive reaction for BDV-N and -P, respectively (Table 1). Four sera showed positive for both, therefore 26 of 171 psychiatric patients showed positive results for at least one of the proteins. One of the healthy controls showed positive for BDV-P, while none of the organic brain disease group showed positive results.

In Western blotting, BDV proteins not fused with GST should be more advantageous than fused proteins to obtain specific results; however, we could not employ unfused proteins because the reaction against BDV-P was extremely reduced when GST was removed. In order to minimize their cross-reactivity to GST, sera were preabsorbed with recombinant GST, and the results of fused proteins were compared with those of GST alone. In some sera, anti-BDV antibodies could not be assessed because of a strong reaction against GST.
GST. Dilution testing constantly showed reasonable results, but inhibition test results were variable (Fig. 3). Most of the sera examined showed some absorption, but the rest showed only little absorption. However, some of them were absorbed when the dilution ratio or the amount of antigen for absorption was changed (data not shown). Mouse monoclonal antibodies showed a strong reaction in Western blotting (Fig. 3). The serum of BDV-infected rat was not examined using this method, but it reacted against BDV-N and -P separated with GST (data not shown). Positive results for [GST]-BDV-N and -P were found in 14 (8%) and 26 (15%) of 171 psychiatric patients, respectively (Table 1), and only one patient showed positive for both antigens. Overall, 39 of 171 psychiatric patients (23%) were positive for at least one antigen. None of nine organic brain diseases showed positive reactions. Healthy controls were not examined by Western blotting.

Comparing the results between the two methods, three and nine sera were concordantly positive for BDV-N and -P, respectively (Table 1). The positive cases by radioligand assay are shown in Table 2 with the results of Western blotting.

The major difference between the two methods may be caused by the form of the antigen. In radioligand assay, antigens are produced in a rabbit reticulocyte lysate system in a way similar to in vivo synthesis without any additives; because the refinement procedure is very simple and the reaction is performed in the liquid phase, the conformational structure should be preserved. In Western blotting, the antigen is fused with GST, denatured into a linear structure, and fixed on the membrane.

Surprisingly, the four sera with the highest indices in the radioligand assay were all negative in Western blotting. The results of dilution and inhibition tests in radioligand assay for these sera (Fig. 1) strongly supported the specificity of the results. Because a conformational epitope is formed by two or more discontinuous peptides, it is reasonable that conformational antibodies cannot be detected by Western blotting. Our results suggest the absolute importance of conformational structure for detection of anti-BDV antibodies. This might explain the part the disagreement in results among several methods. Yet the question remains as to why, under Western blotting, the four sera with the highest indices showed negative but some sera with lower indices showed positive. One possible speculation is as follows: if the sera recognized only a single or a few epitopes in each BDV antigen, and if the epitopes recognized were all conformational, the sera would be negative under Western blotting. In addition, if the conformational antibodies had a higher affinity than the linear ones, they could be produced or bind to the antigens more effectively, resulting in higher indices.

Eleven and 17 sera for BDV-N and -P, respectively, were positive in Western blotting but not in radioligand assay. One of the factors for this disagreement may be low specificity of Western blotting due to anti-GST antibodies or non-specific binding against proteins with the same molecular weight. A second factor might be lowered sensitivity of radioligand assay due to the 1:50 dilution ratio. For BDV-P, all sera above mean plus 2 SD of healthy controls proved positive in an easy dilution test, and this was settled as the cutoff value. Therefore, some positive sera may have been included below mean plus 2 SD. The third factor might be the location of the epitope.

Although major epitopes should be located on the surface of the antigen, epitopes located inside the antigen or in the inner part of oligomers would not have been detected by radioligand assay but could have been by Western blotting, unless conformational. The fourth factor for positivity in blotting but not the assay might be the class of the antibody. Recombinant protein G used in radioligand assay binds specifically to IgG, while anti-human IgG (H+L) used in Western blotting detects not only IgG but also IgM and IgA because light chains are common to them all.

Our results by radioligand assay indicate the importance of conformational structure to detect human anti-BDV antibodies. The additional advantage of this assay is quantitative, including its high throughput. This method may have the potential to become a useful tool for the diagnosis of BDV infection.

This work was supported by Grants-in-Aid for Scientific Research (no. 14207107) and Special Coordination Funds for Promoting Science and Technology, both from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Grant-in-Aid for Community Health and Medical Care from Iehou Association for Promotion of Medical Science.

REFERENCES


