Evaluation of Serological Diagnosis of Borna Disease Virus Infection Using Recombinant Proteins in Experimentally Infected Rats

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ABSTRACT. We produced two recombinant Borna disease virus (BDV) proteins, p40 and p24, by using a baculovirus vector as a diagnostic antigen. Antigenicities of these recombinant proteins were evaluated by immune rabbit sera. Recombinant p40 was a more sensitive antigen than p24 for the detection of antibodies in infected rats. Rats inoculated with BDV within 24 hr after birth showed higher detection rates of viral RNA and viral proteins from the brain than rats inoculated at 4 weeks-old. Depending on the age of infection and the time postinfection, the detection of BDV RNA, protein, or anti-BDV antibody did not always correlate in individuals. We suggest both serological and molecular biological methods are needed in the diagnosis of BDV infection. — KEY WORDS: baculovirus, BDV, diagnosis.

Borna disease in horses has been known as a chronic encephalomyelitis endemic in Germany and several other European countries. Natural infection of Borna disease virus (BDV) has been reported in other vertebrates [16]. Furthermore, recent epidemiological studies have shown suggestive data that BDV may have some relations with neuropsychiatric disorders in humans based on findings of a higher prevalence of anti-BDV antibody and BDV genome among the psychiatric patients than among healthy individuals [2, 12, 17]. Therefore, these findings have been leading to concerns of public health in regards to this potential emergent zoonosis. In Japan, distributions of BDV have been reported among humans, horses, cattle, sheep and cats by means of antibody and viral genome detection [6, 7, 12, 14, 15].

BDV is an enveloped nonsegmented, negative-stranded RNA virus [4, 5]. The BDV genome contains five open reading frames (ORFs), I to V. Viral polypeptides with predicted molecular masses of 40 and 24 kDa corresponding to ORFs I and II, respectively, have been detected in BDV-infected cells and tissues [4, 5].

For serological diagnostic testing of BDV infections, the indirect immunofluorescent antibody (IFA) test utilizing BDV infected cells as antigens has been most commonly used in the epidemiological survey in horses, human and other animals. The IFA titers of anti-BDV antibodies in naturally infected animals are usually very low, often resulting in misleading findings in many epidemiological studies [11, 19]. In an attempt to solve these problems, recombinant BDV proteins recently have been applied to several test systems including enzyme-linked immunosorbant assay and Western blotting in seroepidemiological studies. However, there still have been discrepancies among the results of these studies [9, 12, 17].

To establish the serological diagnosis of BDV infection, we tried to generate two recombinant BDV proteins, p40 and p24 as diagnostic antigens. These antigens were evaluated for their ability to detect antibodies in the sera from experimentally infected rats. The results of anti-BDV antibody detections were compared with those of the antigen and viral genome detections.

cDNA clones corresponding to p40 and p24 were inserted into a baculovirus vector and expressed in insect cells (High Five cells) using BAC-TO-BACTM Baculovirus Expression System (GIBCO BRL, U.S.A.). To produce anti-p40 or -p24 polyclonal antibodies, glutathione-S-transferase (GST) fusion proteins of p40 or p24, which were gifts from Dr. K. Ikuta et al. (Institute of Immunological Sciences, Hokkaido University), were injected into rabbits and their sera were collected.

Antigenicities of these recombinant proteins were examined by the reaction patterns of rabbit antisera described above by IFA test [21]. High IFA titers were obtained in the homologous combinations between rabbit antisera and recombinant proteins (Table 1). Native BDV antigens in persistently infected MDCK cells [8] were detected by rabbit antisera to both p40 and p24 in IFA. Four times higher titer of rabbit anti p24 sera to rp24 than to MDCK/BDV would indicated the different antigenecities between recombinant p24 and native p24. The both antisera exhibited no reactivity with normal High Five and MDCK cell negative controls.

In Western blot (WB) analysis, the p40 and p24 proteins migrated as expected and were detected only by homologous antisera (Fig. 1). We could detect rp40 in the supernatant of the High Five cell cultures by 3 days postinfection (p.i.). However Hsu et al. [10] reported that both recombinant protein (p40 and p24) were secreted into the media from other insect cells (Sf-9) in tissue culture. These might be...
due to the difference of posttranslational modifications between High Five cells and Sf-9. Generally, a soluble antigen is thought to be easy to purify and useful for enzyme-linked immunosorbant assay (ELISA). In addition, our soluble p40 showed no degradation product in WB (Fig. 1). Further application of this soluble p40 antigen is expected for ELISA which is well suited to the screening of multiple samples.

We utilized both recombinant proteins to detect antibodies in sera from infected rats. Adult rats were inoculated intracerebrally with MDCK/BDV cells. Sera were collected from tail-cuts every week and were analyzed for the presence of anti-viral protein antibodies by IFA and WB (Fig. 2). IFA analysis detected high titers (1:400–1:12,800) to anti-p40 antibodies from 2 weeks p.i. through the end of the observation period. However anti-p24 antibodies were detected from 8 weeks p.i. with low titers (maximum titers 1:800). In WB using recombinant proteins, anti-p40 antibody was detected in infected rat sera from 3 weeks p.i., but, the specific bands of p24 were not observed in the same serum (Figs. 1 and 2). The lack of reactivity of infected rat sera to rp24 in WB may be related to the

<table>
<thead>
<tr>
<th>Sera</th>
<th>rp40</th>
<th>rp24</th>
<th>High Five MDCK/BDV</th>
<th>MDCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti p40</td>
<td>6,400</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>25,600</td>
</tr>
<tr>
<td>Rabbit anti p24</td>
<td>&lt;100</td>
<td>12,800</td>
<td>&lt;100</td>
<td>3,200</td>
</tr>
<tr>
<td>Rabbit unimmunized</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
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Recombinant proteins (rp40 and rp24) were expressed in High Five insect cells. MDCK/BDV cells were persistently infected with BDV. Rabbit antisera were raised against each of the two BDV proteins synthesized in *E. coli* as GST fusion proteins. Recombinant proteins were generated as follows: Full-length cDNAs encoding p40 or p24 were cloned into the prokaryotic expression vector pGEX for production of recombinant proteins as described [15]. These two fusion proteins were purified by glutathione Sepharose 4B (Pharmacia Biotech AB, Sweden) and used as immunogens to generate the rabbit anti-p40 or p24 polyclonal antibodies. The cDNA fragment encoding p40 or p24 was excised from the pGEX plasmid by digestion with *Xho*I and *Bam*HI. Thereafter the fragments were ligated into the baculovirus donor vector pFAST BAC1 (GIBCO BRL, U.S.A.). Recombinant baculoviruses were generated by transfection of High Five cells with the respective recombinant Bacmid DNA (GIBCO BRL, U.S.A.) as a source of baculovirus DNA using lipofectin according to the manufacturer’s protocol.

![Fig. 1. Western blotting (WB) of recombinant and native proteins of BDV. To evaluate the proteins, SDS-PAGE and immunoblot analysis were performed. The BDV proteins were stained with positive sera or control sera (see below) followed by a secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG and anti-rat IgG (ZYMED, U.S.A.) and developed with the 4-Chloro-1-Naphthol (Sigma, U.S.A.) in the presence of hydrogen peroxide. Rats were inoculated with BDV intracerebrally within 24 hr after birth. The rats were sacrificed at three weeks later and proteins were extracted from brains using ISOGEN (Nippon Gene, Tokyo) according to the manufacturer’s protocol. For WB, cell lysates from infected and uninfected High Five cells or MDCK cells were dissolved with PBS and stored at -80°C until use. Lanes 1) Rabbit anti-p40 serum 2) Rabbit anti-p24 serum 3) Rabbit unimmunized serum 4) Rat infected serum 5) Rat uninfected serum.](image-url)
sensitivity of WB lower than that of IFA. Besides, since infected rat sera detected native p24 only in infected rat brain by WB, native p24 derived from MDCK/BDV might be slightly different from p24 synthesized in infected rat brain. These results indicate that the recombinant p40 is a more sensitive antigen than this recombinant p24 for the detection of antibodies in infected animals. Our results confirmed the previous findings that the p40 sensitivity is higher than p24 as reported by Briese et al. in rat sera [3] and by Sauder et al. in human sera [17]. It is assumed that p40 is the viral nucleoprotein (N) and p24 is the phosphoprotein (P) based on the corresponding position of ORFs on the viral genome, and the abundance of the respective proteins in infected cells [4, 5, 18]. In other viral infections, the recombinant viral nucleoproteins have been used as target antigens for diagnostic methods because of their antigenic conservation [1, 13, 20].

To evaluate the diagnosis methods of BDV infection in rats, we compared the incidence of the viral antigen (p40 and p24) and RNA (p40 and p24) in brains with antibody response (Table 2). Since the age of the rat at infection might affect the detection of viral RNA or proteins, and antibodies, two groups of rats (newborn animals and 4 weeks-old) were inoculated with BDV. In newborn rats, we could detect the viral antigen and RNA throughout the experiments although the antibodies were undetectable until 6 weeks p.i. In adult rats, we could detect the anti-p40 antibody from 2 weeks p.i. (Fig. 2) although the viral antigens were absent until the late stage of infection. Since the viral RNA was failed to detect from some of antigen positive specimens, the sensitivity of our PCR system seems to be lower than that of the antigen detection. Therefore further examination of our PCR system should be carried out. However our results coincide with previously described reports of epidemiological studies in which there were individuals positive only for BDV RNA and individuals positive only for anti-BDV antibodies in humans, cats and horses [12, 14, 15]. The detection rates of antigen or RNA of BDV were apparently higher in newborn rats than in adult rats. This indicates that BDV replicated very well in newborn rats and was not eliminated in spite of the presence of high titer antibodies. Also, six of eight newborn rats inoculated with BDV showed clinical symptoms such as weight loss and ruffled fur. Two of six diseased rats died at

Table 2. Detection of viral antigen, RNA and antibodies in rats infected with BDV

<table>
<thead>
<tr>
<th>Age i.c.</th>
<th>Weeks</th>
<th>Viral antigen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Viral RNA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Antibody titers&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>after infection</td>
<td>positive/tested</td>
<td>positive/tested</td>
<td>p40</td>
</tr>
<tr>
<td>NB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>3/3</td>
<td>3/3</td>
<td>&lt;100, &lt;100, &lt;100</td>
</tr>
<tr>
<td>NB</td>
<td>6</td>
<td>3/3</td>
<td>3/3</td>
<td>&lt;100, &lt;100, 400</td>
</tr>
<tr>
<td>NB</td>
<td>11</td>
<td>2/2</td>
<td>2/2</td>
<td>12800, 12800</td>
</tr>
<tr>
<td>4 wks&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
<td>0/2</td>
<td>0/2</td>
<td>800, 3200</td>
</tr>
<tr>
<td>4 wks</td>
<td>26</td>
<td>2/2</td>
<td>0/2</td>
<td>1600, 3200</td>
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</tbody>
</table>

Total RNA extraction from rat brain was performed by using ISOGEN according to the manufacturer's protocol. Extracted total RNA was reverse transcribed (RT) by using 200 U of Superscript II RNase H-RT (GIBCO BRL, U.S.A.) in a total volume of 20 µl using BDV p24-specific primer pair (5'-TGACCCAACGATAGACCA-3' at nucleotides 1387–1405 and 5'-GTCCCATTCATC-GTGTGCT-3' at nucleotides 1865–1847) as previously described [15]. The same primer pair was used for amplification of BDV p24 by polymerase chain reaction (PCR). The condition for BDV p24 PCR was as follows: 94°C for 9 min (1 cycle), 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min (10 cycles). The letters denote: a) Newborn (inoculum; infected rat brain homogenates) b) Brain viral antigen was detected by WB with rabbit anti p24 and anti p40 antibody. c) Brain viral RNA was detected by RT-PCR. d) Brain viral RNA was detected by RT-PCR. e) Brain viral RNA was detected by RT-PCR. f) Anti-BDV antibodies titers were determined by IFA.
5 weeks and 8 weeks p.i. On the other hand, in the case of adult rats inoculated with BDV, only one of ten rats showed clinical symptoms (data not shown). Differences of antibody responses, BDV detection and clinical symptoms in newborn and adult groups might be related to the development of immunological responsiveness during aging.

In conclusion, our results confirmed and clarified the following; 1) Two recombinant proteins, p40 and p24, were expressed by a baculovirus vector and showed antigenic responsiveness to monospecific immune sera from rabbit immunized with recombinant GST fusion p24 or p40. 2) rp40 was a more sensitive antigen than rp24 for detection of antibodies in infected rats. 3) To investigate prevalence of BDV infection among natural populations of animals, not only antibody detection but also antigen and RNA testing is needed for diagnosis since the presence of BDV RNA, proteins, or anti-BDV antibody did not correlate in individuals.

Since rats are suspected as one of the reservoirs (personal communication, de la Torre, J.C.), the information in the present study should be useful for epidemiological study to identify a rat as a reservoir animal of BDV.

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REFERENCES