NOTE *Virology*

Antibodies to Borna Disease Virus in Infected Adult Rats: An Early Appearance of Anti-p10 Antibody and Recognition of Novel Virus-Specific Proteins in Infected Animal Brain Cells

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ABSTRACT. The time course for appearance of antibodies to Borna disease virus (BDV) major antigens, p40, p24, p18 and p10 were investigated in BDV-inoculated adult rats by Western blotting. Anti-p10 antibodies were detected in sera as early as anti-p40 and -p24 antibodies at four or five weeks after inoculation. Furthermore, in addition to these major antigens of BDV, the rat serum could detect additional 80-, 58-, 43-, 20-, and 16-kDa proteins in BDV-infected cultured cells and/or animal brain cells by Western blot analysis. Of these proteins, the 20- and 16-kDa proteins were shown to be related to p24 protein by their reactivity with anti-p24 monoclonal antibody. Interestingly, the 58- and 24-kDa were found only in BDV-infected animal brain cells but not in cultured cells. The results in this study could provide a useful information on the mechanism for the viral replication and pathogenesis.

KEY WORDS: animal brain, antibody, Borna disease virus.

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Borna disease virus (BDV) is a neurotropic, negative-strand RNA virus that causes nonpurulent encephalomyelitis in horse and sheep. It has been reported that BDV also naturally infects cattle and cats [4, 13] and experimentally infects rabbits and rats [14, 17]. These animals sometimes show neurological symptoms and movement disorders. Furthermore, recent epidemiological studies suggest that BDV can also infect human and associate with certain psychiatric diseases [3, 20].

Molecular biological analysis has indicated that BDV antigenome consists of at least six open reading frames (ORFs). The ORFs encode nucleoprotein (p40), phosphoprotein (p24), matrix protein (gp18), envelope protein (p56), and a predicted RNA-dependent RNA polymerase (p180) in 5' to 3' order [1, 5, 11, 21]. In addition, a small ORF X, which overlaps p24 ORF, was recently reported to encode a novel protein X (p10) that has an unknown function [15, 23]. Of these proteins, BDV-p40 and -p24 are found as abundant proteins in BDV-infected brain cells of experimentally and naturally infected animals [1]. Furthermore, experimental infection of BDV demonstrated that antibodies against BDV-p40 and -p24 proteins were readily detected in both sera and cerebrospinal fluid of infected hosts [8]. Antibodies against BDV-p10 and -gp18 were also present in sera of adult rats infected with BDV [10, 23]. Previous study demonstrated that appearance of neutralizing antibodies to BDV in infected rats correlates with immunological reactivity to gp18 protein [10]. Detailed investigation of immune response to individual viral proteins is important for understanding how BDV distributes in infected animals as well as development of diagnostic materials for this virus infection.

In this study, to develop a better understanding of immune response to BDV infection, we inoculated BDV to adult rats and determined time course for the appearance of antibodies to each viral protein, particularly anti-p10 antibody, which production in infected animals has not been fully understood yet. Furthermore, we asked any additional BDV-specific antigens expressed in infected animal brain cells by Western blotting using the infected rat serum.

Detection of anti-BDV antibodies in infected rats and time course for appearance of antibodies to each BDV antigen: Three 4-weeks-old Lewis rats were inoculated intracranially with 2×10^2 focus forming unit of virus stock prepared from persistently BDV strain He/80-1-infected C6 cell (C6/BV) [21]. After the inoculation, blood samples were sequentially collected from the tail veins at seven days intervals for 15 weeks. All of rats showed no neurological symptom during the observation period. Anti-BDV antibodies were first detected at 4 weeks post-inoculation (p.i.) in sera of two rats (Fig. 1, Rat 2 and 3) and at 6 weeks p.i. in a rat (Fig. 1, Rat 1) by indirect immunofluorescence assay (IFA) using C6/BV. Antibody titers were reached to 1:12800 at 8 weeks p.i. and were maintained during the observation period in all rats.

Next, we investigated time courses for the appearance of antibodies to individual BDV proteins in the rat sera by immunoblotting assay. Four BDV antigens, p40, p24, gp18 and p10, were expressed in *E. coli* as fusion proteins with glutathione S-transferase. Recombinant proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride (PVDF) membranes. These membranes were blocked with 5% skim milk and incubated with 1:100 dilutions of infected rat sera. After incubation with secondary antibody, presence of specific antibodies in the rat sera was visualized with Konica immunostaining HRP-1000. As shown in Fig. 1, anti-p10 antibodies were detected at 4 or 5 weeks p.i., as well as anti-p40 and -p24 antibodies in all cases, while anti-gp18 antibody was detected at 8 weeks p.i. only in Rat 1 (Fig. 1). However, the antibody titer to gp18 was remarkably lower than those of other antibodies (data not shown). All of BDV-infected rats showed hypersensitivity or aggressiveness, which are specific sign for BDV infection in rat, at 4 to 5

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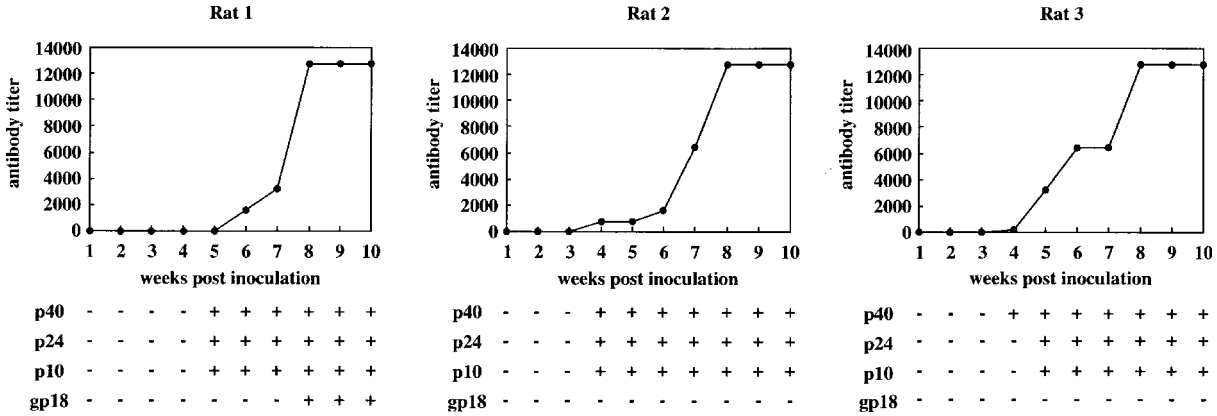

Fig. 1. Time course for appearance of antibodies to BDV antigens in infected rats. Three 4-weeks-old rats were inoculated intracranially with BDV strain He/80-1. Blood samples were collected from tail veins at one-week interval after the inoculation. Antibody titers were determined by IFA using C6/BV cells. Appearance of antibodies to each viral protein was detected by Western blot analysis and showed at below.

weeks p.i.

Analysis of BDV-specific antigens in infected cultured cells and gerbil brain cells: BDV-specific antigens in persistently infected C6 cells and infected animal brain were analyzed by Western blot analysis using the Rat 1 serum described above. Newborn gerbils, which are susceptible to BDV [16], were inoculated intracranially with C6 or C6/BV cell lysates, and the brain samples were collected at 30 days p.i. To detect BDV-specific antigens, C6/BV cells and BDV-infected gerbil brains were separated by 10 or 15% SDS-PAGE and transferred to PVDF membranes. The membranes were reacted with 1:1,000 dilution of Rat 1 serum. Specific signals were visualized by ECL Western blotting detection reagent (Amersham Pharmacia Biotech). As shown in Fig. 2A, the rat serum clearly detected p40 and p24 antigens in both BDV-infected cultured cells and infected gerbil brain (lanes 6 and 8). In addition to the proteins, the serum could also identify several additional BDV-specific proteins. A band with approximately 14.5-kDa protein could correspond to p10 protein, since this band was also detected by anti-p10 polyclonal antibody (Fig. 2A, lane 2 and 4). Furthermore, the serum could demonstrate a few proteins that have not been identified in previous studies. Approximately 16-kDa protein, which was detected in both C6/BV and BDV-infected brain cells, was also reacted with an anti-p24 monoclonal antibody (MoAb) (Fig. 2A, lanes 6, 8, 10 and 12), which recognizes amino acids 41 to 82 of the BDV-p24 protein, indicating that the protein encodes the same coding frame to the p24. In addition, interestingly, approximate 20-kDa protein, which was also detected by the anti-p24 MoAb, was found only in gerbil brain sample but not in BDV-infected cultured cells (Fig. 2A, lanes 8 and 12). Furthermore, using 10% SDS-PAGE, we could find a band with about 58-kDa band only in the gerbil brain sample, in addition to the 80-kDa protein that seems to be a glycosylated forms of envelope protein (Fig. 2B lane 2 and 4). These results indicated a possibility that the BDV can produce several brain cell-specific proteins.

In this study, we investigated antibody responses to BDV major antigens, including anti-p10 antibody, in BDV-infected adult rats and the BDV-specific antigens expressed in infected animal brain. Although it was reported that rats produced anti-p10 antibody after infection of BDV [23], the time course for the appearance of the antibody has not been investigated. In the study, we found that anti-p10 antibody was clearly raised in rat sera as early as the anti-p40 and -p24 antibodies. This result indicated efficient and high expression of p10 protein at early phase of the virus infection. In fact, the p10 antigen was always detected in BDV-infected gerbil or rat brains as well as infected cultured cells by Western blotting or other immunological methods (Fig. 2A) [23]. This feature of the p10 protein suggested that BDV-p10 protein might play an important role in viral proliferation *in vivo*. In addition, the generation of enough antibody titers to p10 protein, as well as to p40 and p24 proteins, was coincident with the appearance of clinical sign in the rats. To date, it has been considered that the appearance of neurological symptoms in BDV-infected rat are assigned to neuronal degeneration induced by cytotoxic T lymphocytes against p40 protein [19] and also that abundant expression of p40 and p24 proteins in infected rat directly lead functional disorder of brain [2]. Thus, it may be likely that expression of p10 or host immune response against this protein could also involve in the pathogenesis of BDV.

Using the infected rat serum, we could find several specific antigens for BDV in infected cultured cells and gerbil brain cells, in addition to the major BDV antigens, such as p40, p24 and p10 (Fig. 2). An 80-kDa protein identified in this study could be a translated glycoprotein of BDV-p56 protein, and previous study has demonstrated that the BDV-p56 protein could produce an additional 43-kDa protein that is generated by cleavage of the 80-kDa protein by a cellular protease [9]. Although we could detect the 43-kDa band on the gels, the band was hidden by a strong intensity of the 40-kDa (Fig. 2B). Since the p56 MoAb or polyclonal antibody has not been produced yet, this serum could be a good tool for study of the p56

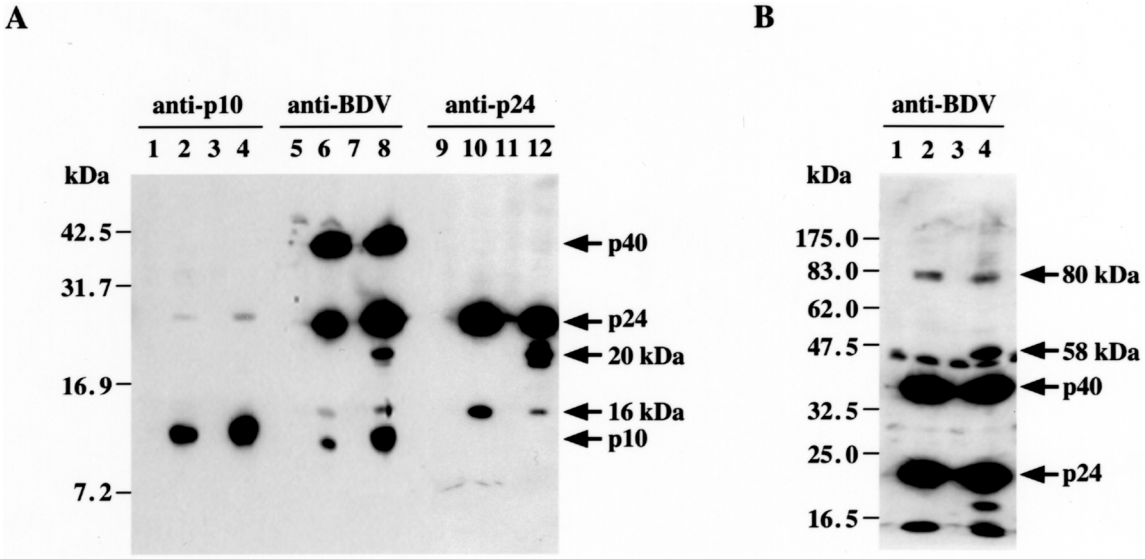

Fig. 2. Western blot analysis of BDV-specific antigens in infected cells and infected gerbil brain with Rat 1 serum. Cell lysates were separated by 15% (A) or 10% (B) SDS-PAGE and analyzed with anti-p10 polyclonal antibody (A; lanes 1 to 4), Rat 1 serum (A; lanes 5 to 8 and B) and anti-p24 MoAb (A; lanes 9 to 12). Lanes: 1, 5, 9, uninfected C6 cells; 2, 6, 10, BDV-persistently infected C6 cells; 3, 7, 11, uninfected gerbil brain; 4, 8, 12, BDV-infected gerbil brain.

protein of BDV.

One of the interesting findings of this study is detection of 20- and 16-kDa proteins that were reacted with anti-p24 MoAb (Fig. 2A), indicating that these proteins share the same coding frame to the p24 protein on the epitope region recognized by the p24 MoAb. This finding may suggest the possibility that several different isoforms of the BDV-p24 protein do exist in the BDV-infected cells. In fact, similar observations have been also found in the other *Mononegavirales*, especially in family *Paramyxoviridae*. In Sendai virus (SeV), P/C mRNA is a bicistronic transcript that encodes both C and P ORFs [7, 18]. C ORF could produce at least four different proteins, C, C', Y1 and Y2 proteins, by initiation readthrough or ribosomal shunt mechanisms [6, 7, 18]. While, SeV P ORF, which corresponds to the p24 ORF of BDV in the genomic structure, could also express P, V, W and D proteins by editing of P gene mRNA [7, 22]. Thus, such polycistronic coding viral mRNAs could be one of the key mechanisms for efficient viral replication. In fact, it has been reported that these proteins of SeV were concerned with regulation of transcription or translation of SeV *in vivo* [8]. Recently, we have found that the 16-kDa protein of BDV is translated from second initiation signal on the p24 ORF (unpublished data). Thus, the 16-kDa protein is not a product resulted from degradation of the p24. Taking together these findings, expression of the 20- and 16-kDa proteins may play important roles for the replication and proliferation of BDV. Particularly, 20-kDa protein was present only in infected gerbil brain but not in persistently infected cultured cell, suggesting that this protein might be play a significant role for pathogenesis in infected brain cells. Similarly, a 58-kDa protein was also found only in the brain cells (Fig. 2B). It could be interesting to examine origin of the 58-kDa protein.

In this study, we could evaluate time course for development of antibodies to individual viral proteins and BDV-specific antigens expressed in infected cultured cells and gerbil brain cells by using serum from BDV-infected rat. The results in this study could be worthy for establishment of diagnostics method for BDV infection and for understanding the viral pathogenesis and its replication in central nerves system of infected animals.

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REFERENCES

1. Bause-Niedrig, I., Pauli, G. and Ludwig, H. 1991. *Vet. Immunol. Immunopathol.* 27: 293–301.
2. Bautista, J. R., Schwartz, G. J., De La Torre, J. C., Moran, T. H. and Carbone, K. M. 1994. *Brain Res. Bull.* 34: 31–40.
3. Bode, L., Riegel, S., Lange, W. and Ludwig, H. 1992. *J. Med. Virol.* 36: 309–315.
4. Caplazi, P., Waldvogel, A., Stitz, L., Braun, U. and Ehrensperger, F. 1994. *J. Comp. Pathol.* 111: 65–72.
5. Cubitt, B., Oldstone, C. and de la Torre, J. C. 1994. *J. Virol.* 68: 1382–1396.
6. Curran, J. and Kolakofsky, D. 1989. *EMBO J.* 8: 521–526.
7. Curran, J. and Kolakofsky, D. 1990. *Enzyme* 44: 244–249.
8. Curran, J., Boeck, R. and Kolakofsky, D. 1991. *EMBO J.* 10: 3079–3085.

9. Gonzalez-Dunia, D., Cubitt, B., Grasser, F. A. and de la Torre, J. C. 1997. *J. Virol.* 71: 3208–3018.
10. Hatalski, C. G., Kliche, S., Stitz, L. and Lipkin, W. I. 1995. *J. Virol*. 69: 741–747.
11. Kliche, S., Briese, T., Henschen, A. H., Stitz, L. and Lipkin, W. I. 1994. *J. Virol.* 68: 6918–6923.
12. Latorre, P., Kolakofsky, D. and Curran, J. 1998. *Mol. Cell. Biol.* 18: 5021–5031.
13. Lundgren, A. L., Lindberg, R., Ludwig, H. and Gosztonyi, G. 1995. *Acta Neuropathol.* 90: 184–193.
14. Ludwig, H., Koester, V., Pauli, G. and Rott, R. 1977. *Arch. Virol*. 55: 209–223.
15. Malik, T. H., Kobayashi, T., Ghosh, M., Kishi, M. and Lai, P. K. 1999. *Virology* 258: 65–72.
16. Nakamura, Y., Nakaya, T., Hagiwara, K., Momiyama, N., Kagawa, Y., Taniyama, H., Ishihara, C., Sata, T., Kurata, T. and Ikuta, K. 1999. *Vaccine* 17: 480–489.
17. Narayan, O., Herzog, S., Frese, K., Scheefers, H. and Rott, R. 1983. *Science* 220: 1401–1403.
18. Patwardhan, S. and Gupta, K. C. 1988. *J. Biol. Chem.* 263: 4907–4913.
19. Planz, O. and Stitz, L. 1999. *J. Virol.* 73: 1715–1718.
20. Rott, R., Herzog, S., Fleischer, B., Winokur, A., Amsterdam, J., Dyson, W. and Koprowski, H. 1985. *Science* 228: 755–756.
21. Schneider, P. A., Hatalski, C. G., Lewis, A. J. and Lipkin, W. I. 1997. *J. Virol.* 71: 331–336.
22. Vidal, S., Curran, J. and Kolakofsky, D. 1990. *J. Virol.* 64: 239–246.
23. Wehner, T., Ruppert, A., Herden, C., Frese, K., Becht, H. and Richt, J. A. 1997. *J. Gen. Virol.* 78: 2459–2466.