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Enzyme-Linked Immunosorbent Assay for Detecting Antibodies to Borna Disease Virus-Specific Proteins

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Borna disease virus is a unique neurotropic RNA virus that causes neurologic disease in a wide variety of animal hosts. We established an enzyme-linked immunosorbent assay for the detection of antibodies to Borna disease virus on the basis of the use of three recombinant viral proteins (recp40, recp23, and recp18). This assay system is more sensitive and rapid than the methods currently used for the serologic diagnosis of infection such as Western blotting (immunoblotting), indirect immunofluorescence test, or immunoprecipitation.

Borna disease virus (BDV) is a nonsegmented, negative-stranded RNA virus (5, 9, 19) that causes a progressive, immune-mediated neurologic disease characterized by behavioral disturbances (20, 24) and movement disorders (28). Although natural infection was originally considered to be restricted to horses and sheep in southeastern Germany, recent studies suggest that BDV infects horses in North America (14), cats in Sweden (21), ostriches in Israel (22), and some human subjects with neuropsychiatric disorders in Europe and North America (3, 4, 10, 26). The diagnosis of infection is based on the appearance of a clinical syndrome consistent with disease and the presence of serum antibodies that detect viral proteins in infected cells by an indirect immunofluorescence test (IFT) (25), Western blotting (WB; immunoblotting), or immunoprecipitation (IP) (20). These methods are cumbersome and difficult to use for large surveys of human and livestock populations.

The BDV genome has been cloned to reveal antisense information for five open reading frames (6, 9). From 5′ to 3′ on the antigenome, the open reading frames are p40, p23, gp18, p57, and pol. Proteins p40, p23, and gp18 have been identified in infected cells and tissues: p40 and p23 are expressed at high levels in vitro and in vivo and are found in the nucleus and cytoplasm of an infected cell (1); gp18 is a membrane-associated glycoprotein that is expressed at lower levels (16). Expression of p57 or pol has not yet been confirmed. We have expressed p40, p23, and gp18 as recombinant proteins and established a sensitive, specific enzyme-linked immunosorbent assay (ELISA) for analyzing immunoreactivity to BDV. This system provides a convenient tool for diagnosing disease, determining the prevalence of infection in animal and human populations, and mapping the antigenic determinants of the immune response in infected hosts.

MATERIALS AND METHODS

Infection of animals and cultured cells. Six-week-old Lewis rats (Charles River) were infected intranasally with 6×10^4 focus-forming units of BDV strain He/80-1 (7, 27). C6 cells were persistently infected with BDV He/80-1 (C6BDV) (8). Rabbit fetal glial cells were infected with BDV He/80-1 at a multiplicity of 1 focus-forming unit per cell and were then passaged once before use in IFTs. BDV strain He/80 was originally isolated from infected horse brain and was passaged twice in rabbits, three times in rabbit fetal glial cells, and twice in Lewis rats (13). BDV strain He/80-1 was passaged four additional times in Lewis rats and was used for infection of animals and cell lines.

Generation of recombinant proteins (recp40, recp23, and recp18). Full-length cDNAs encoding p40, p23, or gp18 were cloned into the prokaryotic expression vector pET15b (Novagen) for the production of recombinant proteins. pBDV-40 in pcDNA II (23) was amplified with the primers p40Xho I (5′-CCCTCGAG GACCAAGATTT-3′) and Sp6 (20mer; Promega Corp.). pBDV-23 in pBluescript SKII$^+$ (29) was amplified with the primers p24Nde I (5′-AGAATCATAT GGCAACGCGACCATC-3′) and T7 (20mer; Promega). PCR was performed with *Taq* polymerase (Perkin-Elmer Cetus Corp.) according to the manufacturer's protocol. Products amplified from pBDV-40 and pBDV-23 were extracted with phenol-chloroform, precipitated, and digested with *Bam*HI and either *Xho*I (pBDV-40) or *Nde*I (pBDV-23) (Promega Corp.). pBDV-gp18 in pBluescript SKII$^+$ (16) was digested with *Xho*I and *Bam*HI. Digested fragments were purified by agarose gel electrophoresis (USBioclean; U.S. Biochemicals) and cloned into pET15b (Novagen). Protein expression in plasmid-containing *Escherichia coli* cells was induced by the addition of isopropyl-β-thiogalactopyranoside (1 mM) for 3 h at 37°C. Proteins (recp40, recp23, and recp18) were purified by nickel-chelate affinity chromatography according to the manufacturer's instructions (Novagen). Purification was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and antigenicity was confirmed by WB with sera from infected rats. Proteins were dialyzed against 150 mM NaCl and 2.5 mM $CaCl_2$ and were digested with biotinylated thrombin (1 U/mg of recombinant protein; Novagen) overnight at room temperature. Thrombin was removed by using streptavidin-agarose (Novagen) according to the manufacturer's protocol. Protein concentrations were estimated by a Bio-Rad protein assay according to the manufacturer's instructions.

Antibodies to BDV and recombinant BDV proteins. Sera were collected from infected rats at the time of sacrifice or by tail bleeding at 2-week intervals after inoculation with BDV. Antibodies to recp40 and recp23 were each produced in two rabbits. Animals were injected subcutaneously with 25 μg of protein in Freund's complete adjuvant and were then boosted 3 weeks later subcutaneously with 25 μg of protein in Freund's incomplete adjuvant. After 6 weeks some animals received an additional subcutaneous injection of 25 μg of protein in Freund's incomplete adjuvant. Blood was collected at 2-week intervals during weeks 7 through 14 for detection of antibodies by WB and ELISA.

IFT. Rabbit fetal glial cells were processed for titration of serum antibodies against BDV by the immunohistochemical methods of Pauli et al. (25). Briefly, infected and noninfected cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS), permeabilized with 1% Triton X-100 in PBS, and blocked with 1% fetal bovine serum (FBS) in PBS. After incubation with sera diluted in 1% FBS in PBS, cells were incubated with fluorescein-conjugated goat anti-rat immunoglobulin G (IgG) and IgM or goat anti-rabbit IgG (Sigma) diluted 1:200 in 1% FBS in PBS and were then examined by fluorescence microscopy. The IFT titer for each serum sample was determined to be the endpoint dilution at which specific immunofluorescence was detected.

SDS-PAGE, WB, and IP. For WB, lysates from infected and noninfected C6 cells were prepared as described by Bause-Niedrig et al. (1). Proteins from these lysates (30 μg) and recombinant BDV proteins (250 ng) were subjected to SDS–12% PAGE (18) and were then transferred to nitrocellulose membranes (Schleicher & Schuell) (30). Membranes were incubated at room temperature

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first with WB diluent (0.5% nonfat dry milk [Carnation] and 0.05% Tween 20 [Fisher] in TBS [Tris balanced saline; 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl]) for 1 h and then overnight with various dilutions (1:10 to 1:2,000) of rat sera or monospecific rabbit sera in WB diluent. Membranes were washed three times in TBS, incubated for 2 h with the appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-rat IgG and IgM or goat anti-rabbit IgG; Sigma) diluted 1:500 in WB diluent, washed five times in TBS, and then incubated with hydrogen peroxide and 4-chloro-1-naphthol (Pierce) according to the manufacturer's instructions. The methods for synthesis and analysis of radiolabeled BDV proteins and immunoprecipitation have been described previously (19). Briefly, plasmid clones pBDV-gp18, pBDV-23, and pBDV-40 were linearized and used as templates for in vitro transcription and translation of [^{35}S]methionine-labeled proteins. After precipitation with rat or rabbit sera and protein A-Sepharose (Sigma), proteins were analyzed by SDS-PAGE and autoradiography.

ELISA. Ninety-six-well, Immulon I microtiter plates with lids (Dynatech Laboratories) were coated overnight at 37°C with 10 ng of recombinant protein per well in 100 μl of borate buffer (100 mM boric acid, 50 mM sodium borate, 75 mM sodium chloride [pH 8.4]). Plates were washed three times with washing buffer (0.05% Tween 20 in PBS) and were incubated for 1 h at 37°C with ELISA diluent (0.5% bovine serum albumin [BSA] fraction V [U.S. Biochemicals] in washing buffer). Twofold serial dilutions of sera were prepared in ELISA diluent; 100 μl of serum diluted from 1:250 to 1:500,000 was then added to each well, and the plates were incubated for 2 h at 37°C. The plates were washed three times with washing buffer. Next, 100 μl of horseradish peroxidase-conjugated goat anti-rat IgG and IgM (Sigma) diluted 1:5,000 in ELISA diluent was added to each well and the plates were incubated for 1 h at 37°C. After the plates were washed five times, 100 μl of substrate solution was added to each well. Substrate solution consisted of 9.9 ml of 100 mM sodium acetate adjusted to pH 6.0 with 100 mM citric acid, 100 μl of 10 mg of 3,3′,5,5′-tetramethylbenzidine (Sigma) per ml in dimethyl sulfoxide, and 1.5 μl of 30% hydrogen peroxide (Fisher). After incubation in the dark at room temperature for 30 min, the reaction was stopped by the addition of 50 μl of 25% sulfuric acid (Sigma) to each well. The A_{450} was determined for each well with a microplate reader (Thermo max; Molecular Devices). Negative control wells, without primary antisera, were used for calibration. The ELISA titer for each serum sample was defined as the endpoint dilution that yielded an optical density of 0.3.

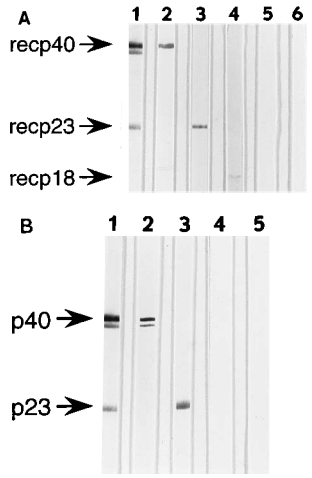

FIG. 1. WB analysis of native and recombinant proteins with monospecific antisera to recombinant proteins and sera from infected rats. Recombinant viral proteins and lysates from infected C6BDV or noninfected C6 cells were size-fractionated and screened by WB. (A) Sera from infected and noninfected rats were used to detect native or recombinant proteins. Lane 1, C6BDV lysate; lane 2, recp40; lane 3, recp23; lane 4, recp18; lane 5, C6BDV lysate; lane 6, recp40, recp23, and recp18. Lanes 1 to 4 were treated with serum from an infected rat; lanes 5 and 6 were treated with serum from a noninfected rat. (B) Monospecific antisera were used to detect BDV-specific proteins. C6BDV lysates (lanes 1 to 3) and C6 lysates (lanes 4 and 5) were incubated with serum from an infected rat (lanes 1 and 4), anti-p40 rabbit serum (lane 2), anti-p23 rabbit serum (lane 3), and pooled anti-p40 and anti-p23 sera (lane 5).

RESULTS

Production of recombinant viral proteins and monospecific antisera to recombinant viral proteins. Full-length coding sequences for p40, p23, and gp18 were expressed in *E. coli*, and recombinant proteins were purified. The protein yields in 100 ml of bacterial culture were as follows: recp40, 1 mg; recp23, 500 μg; and recp18, 50 μg. Recombinant proteins were analyzed by SDS-PAGE. A predominant band of the expected molecular weight was observed for each protein and was tested for antigenicity by WB with sera from BDV-infected and noninfected rats (Fig. 1A). Recombinant proteins were detected by sera from BDV-infected rats but not by sera from noninfected rats. Recombinant proteins recp40 and recp23 were used to produce antibodies in rabbits. The production of antibodies was monitored by ELISA. Rabbits were sacrificed when the ELISA titer reached 1:500,000 (week 16 of immunization). The specificities of the antisera were then tested by WB with lysates from infected cells and recombinant proteins (Fig. 1B). Antisera were monospecific: rabbits immunized with recp40 produced antibodies that reacted only with p40 and recp40; rabbits immunized with recp23 produced antibodies that reacted only with p23 and recp23. At week 16 of immunization, the titers in the antisera were also determined by IFT. Antisera to recp40 and recp23 had IFT titers of 1:50,000 and 1:100,000, respectively.

Specificity and sensitivity demonstrated in the BDV ELISA systems. In order to establish a sensitive and specific ELISA for all three recombinant BDV proteins, the optimal antigen concentration was determined by checkerboard titration of positive and negative sera versus various antigen concentrations. For each protein, the concentration that resulted in the most linear response was 10 ng per well. The sensitivity of the ELISA system for each recombinant protein was established with sera from infected rats known to be reactive by IFT, IP, and WB. For each of the proteins, 100% of sera that had been found to be positive by other methods were also positive by ELISA. Specificity was tested with sera from 15 noninfected rats. Each ELISA proved to be highly specific for the detection of antibodies to BDV proteins: recp40 ELISA with noninfected rat sera showed 80% specificity at a 1:500 dilution or 100% specificity at a 1:2,000 dilution, recp23 ELISA showed 93% specificity at a 1:250 dilution and 100% specificity at a 1:1,000 dilution, and recp18 ELISA showed 100% specificity at a 1:250 dilution. Figure 2 shows a representative ELISA with recp40 as the target antigen. Various dilutions of sera from chronically infected and noninfected rats were tested with 10 ng of either recombinant protein or BSA per well. No nonspecific background reactivity was observed at serum dilutions of 1:500 or higher (Fig. 2). Results were similar when recp23 and recp18 were used as target antigens (data not shown).

Analysis of immunoreactivities to viral proteins by IFT, WB, IP, and ELISA in sera from infected rats. Adult rats infected intranasally with BDV did not display abnormal behaviors prior to the fourth week postinfection (predisease [PD]). At 4 to 6 weeks postinfection, in the acute phase of disease (AD), animals had hyperactivity, weight loss, disheveled fur, dystonic posture, and hind limb paresis. At 8 to 15 weeks postinfection, the signs of disease stabilized: there was no additional weight loss, hyperactivity diminished, and paresis did not progress. This chronic phase of the disease (CD) persisted for the lives of the animals. Sera were collected from adult infected rats between 3 and 15 weeks after infection with BDV and were analyzed for the presence of antibodies to viral proteins by four different methods: IFT, WB, IP, and ELISA (Table 1).

IFT allowed detection of antibodies to BDV in both AD rats and CD rats. In AD rats, the titer was between 1:20 and 1:200, whereas in CD rats, the titer was between 1:10,000 and 1:20,000. Sera from PD rats were not reactive by IFT. WB with lysates from infected cells or recombinant proteins and IP with proteins translated in vitro yielded identical results: sera from

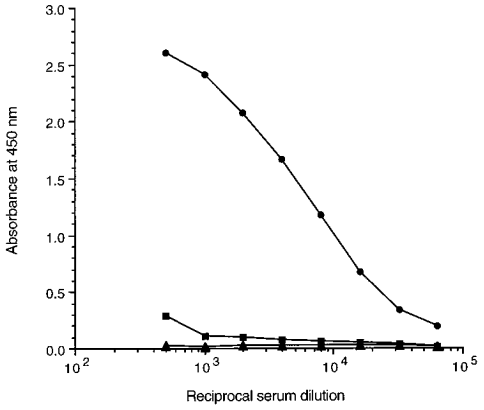

FIG. 2. Infected rat serum reacts with recp40 in ELISA. ELISA was performed with 10 ng of recp40 or BSA per well as described in Materials and Methods. Circles, recp40 and serum from chronically infected rat; squares, recp40 and serum from a noninfected rat; triangles, BSA and serum from a chronically infected rat.

CD animals were reactive with p40, p23, and gp18; sera from AD rats detected only p40 and p23; sera from PD rats did not react with p40, p23, or gp18. ELISA detected antibodies reactive with p40, p23, and gp18 in sera from all CD and AD rats (Table 1). In PD rats, ELISA only detected antibodies reactive with p40 and p23; immunoreactivity with gp18 was below the specificity of the assay (Table 1).

The time course for the appearance of antibodies to BDV proteins in sera was determined by ELISA. Sera collected at regular intervals from adult infected rats were tested in the recp40, recp23, and recp18 ELISA systems. The titers of antibodies to all three proteins increased throughout the period of observation from weeks 4 to 15 postinfection (Fig. 3).

DISCUSSION

Three recombinant BDV proteins, recp40, recp23, and recp18, were expressed and used as immunogens for the production of monospecific sera in rats or rabbits. Results for two of these antisera, directed against recp40 and recp23, are reported here. Results for antisera to recp18 have been reported elsewhere (12). These three recombinant proteins were detected by sera from infected rats (Fig. 1A) and by monoclonal antibodies to purified native proteins (17). Monospecific antisera to the recombinant proteins were immunogen specific as determined by WB (Fig. 1B) and detected proteins in infected cells by IFT.

ELISA systems were established on the basis of recombinant proteins; these systems have several advantages over the methods currently used for the detection of BDV-specific antibodies, including IFT, WB, and IP. Although IFT is widely accepted as a method for diagnosing BDV infection and determining the titers of antibodies to the virus, it has two disadvantages. First, IFT does not define the viral protein(s) responsible for immunoreactivity. Second, as shown here, IFT titers are 10- to 100-fold less sensitive than ELISA for the detection of antibodies to p40 or p23. This relative insensitivity resulted in the failure of IFT to show evidence of infection in PD rats (Table 1). WB and IP allowed for the detection of antibodies to individual viral proteins but were also less sensitive than ELISA. Sera from PD rats were not reactive by either WB or IP.

For diagnostic purposes, the recp40 ELISA is the most sensitive method for the detection of antibodies in infected animals. Antibodies to recp40 were present prior to disease onset and were present at higher titers than antibodies to recp23 or recp18. Although the recp23 ELISA was also positive for PD and AD rats, the recp18 ELISA was not. Because high-titer antibodies to gp18 only appear in CD rats, the recp18 ELISA may be used to estimate the duration of infection. Low antibody titers to recp18 are not due to the lack of glycosylation on this recombinant protein because similar ELISA titers were found with native gp18 antigen (11). Failure to produce a high-titer antibody response to recp18 may be due to the lower levels of expression of this protein than those of p40 or p23 (15).

Growing recognition that BDV has a broader species and geographic range than previously appreciated suggests the importance of designing sensitive, reliable assays for the determination of infection. The ELISA systems described here provide inexpensive, rapid methods for the determination of BDV serology. In contrast to IFT, WB, and IP, which require at least 2 days for completion and are not well suited to the screening of multiple samples, ELISA allows analysis of hundreds of serum samples in several hours with only minimal equipment. Plates coated with these proteins have been stable for ELISA for up to 1 month at room temperature and thus are practical for use in remote laboratories. In addition to serving as a tool for the clinical diagnosis of and determination of the epidemiology of BDV infection, the BDV ELISA should be a useful tool for studies in immunopathogenesis and virus biology. For example, antigen-binding sites on p40 and p23 were recently mapped by ELISA with sera from infected animals and monoclonal antibodies to BDV proteins (15).

Dependent on the population studied and the methods used for analysis (WB, IP, or IFT), the prevalence of antibodies reactive with BDV proteins in patients with neuropsychiatric disorders has been estimated to be between 4 and 23% (2). Variability between laboratories could be due to differences in the populations analyzed, antigen preparations, or experimental technique. The BDV ELISA based on recombinant proteins provides a standardized method for investigating human immunoreactivity to this neurotropic infectious agent.

TABLE 1. Detection of BDV-specific antibodies in sera from infected rats by different methods

Serum	WB			IP[a]			Reciprocal ELISA titer[b]			Reciprocal IFT titer
	recp40	recp23	recp18	p40	p23	p18	recp40	recp23	recp18	
PD (3–4 wk pi[c]; $n = 15$)	−	−	−	−	−	−	2,388 ± 256	904 ± 181	163 ± 5[d]	<10
AD (4–6 wk pi; $n = 18$)	+	+	−	+	+	−	3,217 ± 829	2,644 ± 20	279 ± 19	20–200
CD (10–15 wk pi; $n = 14$)	+	+	+	+	+	+	291,889 ± 56,590	76,527 ± 13,309	4,680 ± 1,467	10,000–20,000

[a] In vitro-translated proteins.
[b] Values are mean ± standard error of the mean titer.
[c] pi, postinfection.
[d] Nonspecific. Value below the level of specificity of the recp18 ELISA (1:250).

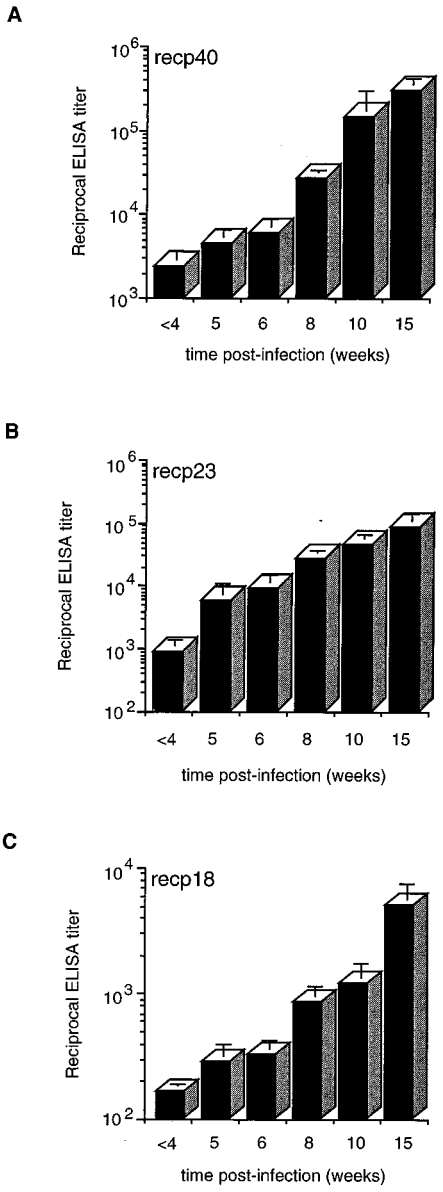

FIG. 3. Time course for the appearance of antibodies to BDV proteins. Sera were collected at different times postinfection and were assayed by ELISA for antibodies to recp40 (A), recp23 (B), and recp18 (C). Error bars represent standard errors of the means. The numbers of animals analyzed at each time point were 15 at <4 weeks, 6 at 5 weeks, 12 at 6 weeks, 4 at 8 weeks, 5 at 10 weeks, and 9 at 15 weeks.

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