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Induction of Protection against Borna Disease by Inoculation with High-Dose-Attenuated Borna Disease Virus

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Borna disease is a chronic neurological disease caused by an enveloped negative-strand RNA virus (BDV). Experimental disease can be reproduced in rats with brain homogenates derived from infected animals or with virus derived from infected cells in culture. The virus replicates in cultured cells without evidence of cytopathic effect or production of significant levels of cell-free virus. Borna disease is caused by an immunopathological response to viral infection of neural cells. To further investigate the pathogenesis of Borna disease, rats were inoculated with different doses of BDV attenuated by culture in MDCK cells. Low doses of attenuated BDV (10^2–10^4 $TCID_{50}$) resulted in typical clinical disease and severe encephalitis; however, the lag period between inoculation and disease was considerably longer than that with virulent BDV. In contrast, animals inoculated with a high dose of attenuated BDV (10^5–10^6 $TCID_{50}$) did not develop clinical disease, although a mild encephalitic response was present that did not progress beyond the mild encephalitis. Animals inoculated with a high dose of BDV developed high titers of anti-BDV antibody and were protected against virulent challenge. Protection was correlated with the rapid induction of an immune response in the animals and the lack of any biologically detectable virus in the CNS.

INTRODUCTION

Borna disease (BD) is a chronic encephalitis of horses and sheep (Heinig, 1969) that is caused by an enveloped negative-strand RNA virus (BDV) (VandeWoude *et al.*, 1990; Lipkin *et al.*, 1990; Briese *et al.*, 1992; Richt *et al.*, 1991, 1992, 1993; de la Torre *et al.*, 1990; McClure *et al.*, 1992; Thierer *et al.*, 1992; Pyper *et al.*, 1993; Cubitt *et al.*, 1994; Briese *et al.*, 1994). Animals with active BD have high levels of infectious virus in the brain. Experimentally the disease can be reproduced by inoculation of various species of animals with brain homogenate from infected horses (Narayan *et al.*, 1983a,b). The virus is propagated in brain by intracerebral (IC) inoculation of rats (Narayan *et al.*, 1983b) or by inoculation of fetal brain cell cultures (Herzog and Rott, 1980). Nonneural cells such as MDCK cells can be infected by cocultivation with infected fetal rabbit brain cells. The MDCK cells become persistently infected and do not develop cytopathic effect. Virus obtained from lysed MDCK cells has attenuated pathogenicity in rats.

In weanling rats BDV replicates only in cells in the nervous system (Herzog *et al.*, 1984). Inoculation of virus intravenously fails to cause infection, and the virus is cleared rapidly from circulation (Carbone *et al.*, 1987). However, when inoculated intranasally (Morales *et al.*, 1988) or in the footpad (Carbone *et al.*, 1987) BDV enters peripheral nerves and is transported within neuronal processes to ganglia and the brain where replication occurs. The virus replicates preferentially in neurons of the limbic system of the brain and the rate of replication is a function of the dose of virus in the inoculum (Carbone *et al.*, 1987). In addition to neurons, astrocytes are also frequently infected, particularly after the acute phase of disease (Carbone *et al.*, 1989, 1991). Rats inoculated IC with undiluted infectious brain homogenate (containing 10^4 $TCID_{50}$) have detectable virus by Day 6, whereas those inoculated with low doses (10 $TCID_{50}$) require 3 to 4 weeks for detectable virus replication. In both cases, once begun, virus replication continues exponentially to high titers in the brain (10^6 $TCID_{50}$/ml of 10% brain homogenate). Replication of BDV in the CNS elicits a severe inflammatory response characterized by an influx of CD4+ and CD8+ T-lymphocytes, B-lymphocytes, and macrophages (Deschl *et al.*, 1990), frequently accompanied by loss of infected brain cells. Encephalitis becomes apparent at the time of exponential virus replication, beginning about 14 days following inoculation with 10^4 $TCID_{50}$ of brain homogenate and 4–6 weeks following 10 $TCID_{50}$ (Carbone *et al.*, 1987). Animals surviving acute BDV encephalitis have extensive atrophy of the cerebral cortex and a reduced virus burden in the brain (Carbone *et al.*, 1987). Lesions and disease are preventable by

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treatment of infected animals with drugs or antisera that prevent inflammation (Narayan *et al.,* 1983b; Stitz *et al.,* 1989, 1992); these treatments are effective only if administered no later than 1 day after virus inoculation. Infected, treated animals become virus carriers, supporting extensive replication in the brain. These animals readily develop disease after adoptive immunization with BDV-specific T-cells (Narayan *et al.,* 1983b; Richt *et al.,* 1989). Thus, the inflammatory disease is caused by the cell-mediated immune response to the virus and this is dependent on expression of viral antigens in CNS cells during exponential virus replication in the brain.

In this study, cell-culture-attenuated BDV was examined in weanling rats for its potential to cause disease and to generate immune responses in infected animals. Inoculation of low doses paralleled virulent virus infection although pathological changes, virus replication, and the immune response were delayed for a month. It was found that inoculation of a high dose of attenuated BDV resulted in a rapid, vigorous immune response without virus replication. The animals were protected against challenge with virulent BDV.

MATERIALS AND METHODS

Virus

Persistently BDV-infected Madin Darby canine kidney (BDV/MDCK) cells were used as a source of virus (Herzog and Rott, 1980). To obtain stock virus, confluent monolayers of BDV/MDCK were prepared by cultivation of the cells in MEM with 10% FBS. Approximately 10^9 cells were suspended in 20 ml of MEM plus 5% FBS and sonicated for 30 sec at 4°. This stock virus was stored in aliquots at −70° and had a titer of 5×10^7 $TCID_{50}$/ml. Virus stocks were assayed for infectivity in fetal rabbit glial cultures prepared from brains of 16-day-old fetal rabbits and stored in liquid nitrogen (Herzog and Rott, 1980). The glial cells were cultivated in MEM plus 10% FBS in chamber slides, inoculated with dilutions of test material, and maintained for 7–10 days at 37°. The cultures were then fixed in acetone at 4° and BDV antigens were detected using an immunofluorescence assay (IFA) (Herzog and Rott, 1980). Sera from BDV-infected rats or Bo18, a monoclonal antibody to the BDV 38-kDa protein (Haas *et al.,* 1986), were used for the IFAs.

Rats

Four-week-old outbred CD rats (Charles River) were used throughout these experiments. The animals were lightly anesthetized with methoxyfluorane and inoculated IC with 50 μl virus material using a 23-gauge needle. The animals were killed at various times after inoculation by an overdose of methoxyfluorane followed by exsanguination.

Histopathology and Immunohistochemistry

Brain tissue was fixed in 10% buffered formalin and embedded in paraffin. Routine hematoxylin and eosin staining was performed on 5-μm sections. Four sagittal sections of the brain were examined on each rat. Pathological changes were graded as mild (occasional perivascular cuffs and only mild cortical hypercellularity), moderate (numerous perivascular cuffs, cortical hypercellularity, and diffuse parenchymal inflammation especially in cerebral cortex and hippocampus), or severe (large number of perivascular cuffs, severe diffuse cortical and hippocampal inflammation, and neuronal degeneration and loss). An avidin–biotin–peroxidase method (Vector Laboratories, Burlingame, CA) was used with the Bo18 monoclonal antibody (Haas *et al.,* 1986) to detect BDV-infected cells and with anti-GFAP (DAKO) to detect astrocytes. Lectin histochemistry using the biotinylated lectin *Ricinus communis* agglutinin-I (RCA-I) (Vector) was used to identify macrophages as described (Sharma *et al.,* 1992).

Measurement of virus titers in brain

A 10% rat brain homogenate was prepared in MEM–5% FBS using a Dounce homogenizer. Dilutions of the homogenate were used to infect fetal rabbit glial cells in chamber slides. After incubation for 7–10 days, the cells were fixed, and BDV antigens were detected using IFA (Herzog and Rott, 1980).

Measurement of anti-BDV antibody titers in infected rats

Persistently infected MDCK cells were grown on Teflon-coated slides. Antibody titers were measured in IFAs using 10-fold dilutions of sera from infected animals and determining the greatest dilution at which the infected MDCK cells could be detected.

In situ hybridization

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in a graded alcohol series and pretreated with 0.2 *N* HCl and 25 μg/ml proteinase K. The tissues were then acetylated, dehydrated through a graded alcohol series, and air-dried as previously described (Zink *et al.,* 1990). *In situ* hybridization was performed using the 700-bp cDNA clone B8 (Vande Woude *et al.,* 1990) of BDV that was radiolabeled by random priming using ^{35}S-labeled dCTP (Amersham Corp., Arlington Heights, IL). B8 is a cDNA clone that encodes the 24-kDa protein. Radiolabeled DNA (0.2 μg/ml) was denatured by heating and placed over the pretreated tissues. After hybridization (16 hr at RT), the slides were washed, dehydrated, dipped in NTB3 autoradiographic emulsion (Eastman Kodak Co., Rochester, NY), air-dried, and devel-

TABLE 1

TEMPORAL ANALYSIS OF RATS INFECTED WITH LOW-DOSE (10^2 $TCID_{50}$) AND HIGH-DOSE (10^6 $TCID_{50}$) ATTENUATED BDV[a]

	Days	No. of rats	Virus titer[b]	Viral antigen[c]	Viral RNA[d]	Ab titer[e]	Pathology[f]
Low dose							
	17–20	4	0	N.D.	N.D.	L	N.D.
	30–40	2	10^4	+	+	L	−
	44–47	1	10^3	++	+	L	−
		1	10^4	++	+	L	−
		2	10^5	++	+	L	−
		1	10^6	++	+	L	−
	53	1	10^4	++	+	H	+++
		1	10^4	++	+	L	−
		1	10^5	++	+	H	+++
		1	10^5	++	+	L	−
	63	2	10^5	+	+	L	−
	68	3	10^5	++	+	H	+++
		2	10^5	++	+	L	−
		1	10^3	++	+	H	+++
High dose							
	7	1	0	+	−	L	N.D.
	11	1	0	+	−	H	+
	17	3	0	−	−	H	N.D.
	44	2	0	−	−	H	+
	53	3	0	−	−	H	+
	68	3	0	−	−	L	N.D.
		1	0	−	−	H	N.D.

[a] Rats were inoculated IC with low-dose (10^2 $TCID_{50}$) or high-dose (10^6 $TCID_{50}$) attenuated BDV. At various times after infection they were sacrificed for determination of viral titer, presence of viral antigen and RNA in the brain, antibody titer, and pathology.

[b] Fetal rabbit glial cells in chamber slides were infected with dilutions of brain homogenate from infected rats. After 7–10 days infected cells were identified by IFA.

[c] Viral antigen was detected in brain sections by immunohistochemical techniques. +, antigen was detected; −, antigen was not detected; ND, not determined.

[d] Viral RNA was detected in brain sections by *in situ* hybridization using a BDV-specific probe. +, BDV RNA was detected; −, BDV RNA was not detected.

[e] Anti-BDV antibody titers were determined by IFA using dilutions of sera from infected rats and persistently infected MDCK cells that are uniformly infected. L, ≤1:100; H, ≥1:1000.

[f] −, no or minimal inflammation; +, mild inflammation; +++, severe inflammation.

oped after 3 to 10 days exposure in the dark. Slides were examined by light microscopy; the presence of viral RNA was indicated by silver grains over cells.

Analysis of BD viral RNA

Extraction of RNA. Total RNA was prepared from rat brains using the method of Chomczynski and Sacchi (1987).

Northern analysis. Ten micrograms of total RNA was electrophoresed in a 1% agarose–formaldehyde gel and transferred to Hybond-N nylon membrane (Amersham). After uv-crosslinking, the membrane was prehybridized at 42° in hybridization buffer containing 50% deionized formamide, 6× SSC, 20 μg/ml tRNA, 0.05 *M* HEPES (pH 7.0), 5× Denhardt's, 1% SDS, 100 μg/ml heparin, 1% glycine, and 20 μg/ml sonicated denatured salmon sperm DNA. The membrane was then hybridized at 42° with 2.5 × 10^6 cpm/ml of ^{32}P-end-labeled oligonucleotide 247 (5′-GCCTCCTGACTAATTCAAGCAATGTCAGCCGGAGGTC-GGAACTC-3′). Oligonucleotide 247 is an antisense oligonucleotide derived from the sequence encoding the 24-kDa protein. After overnight hybridization, the membrane was washed twice at 42° with 6× SSC and then autoradiographed.

Semiquantitative RT-PCR. To detect rare species of RNA, a sensitive RT-PCR technique was used in which a labeled oligonucleotide was used in the PCR reaction for enhanced detection (Saltarelli *et al.*, 1993). For reverse transcription, 2.5 μg total RNA was denatured at 68° for 10 min and placed on ice. The reaction contained 1× PCR reaction buffer (10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin), 125 μ*M* dATP, 125 μ*M* dCTP, 125 μ*M* dGTP, 125 μ*M* dTTP, 100 units/ml RNasin (Promega), 100 units/ml AMV reverse transcriptase (Life Sciences), and 50 μg/ml $pd(N)_6$ (Pharmacia) in a volume of 50 μl. Samples were incubated at 45° for 2 hr. Two BDV-specific oligonucleotides derived from the sequence encoding the 24-kDa protein were used as primers for PCR.

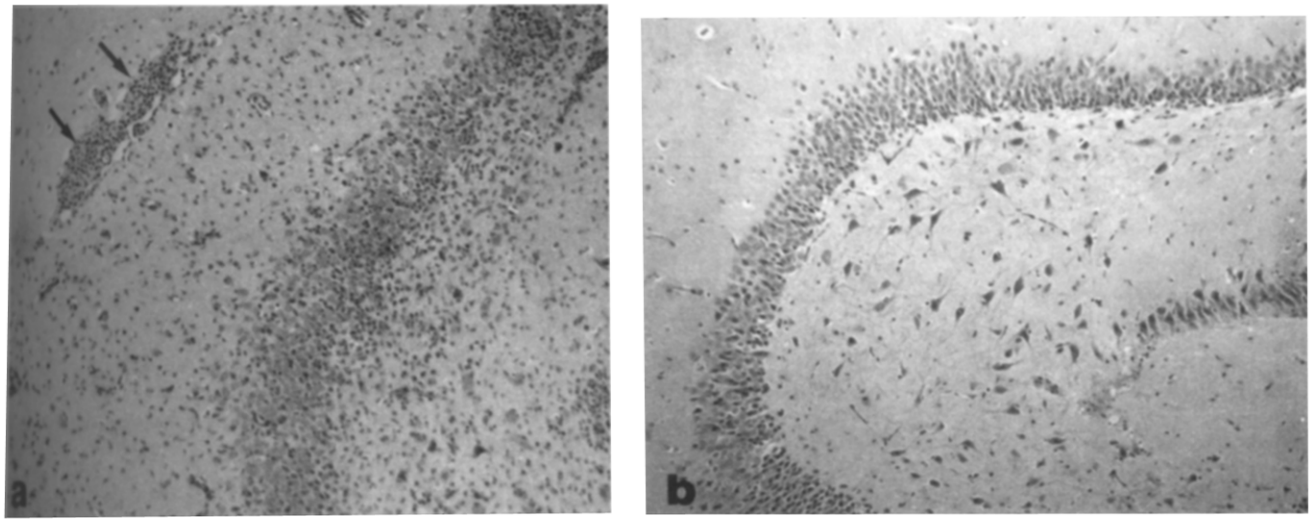

FIG. 1. Sections of brain tissue (hippocampus) from rats inoculated with 10^3 (a) or 10^6 (b) $TCID_{50}$ BDV. Brain tissue from animals inoculated with the lower dose (a) had severe encephalitis, with marked diffuse and perivascular (arrows) infiltrates of lymphocytes. In contrast, the animals inoculated with the higher dose of virus (b) had only mild encephalitis with a minimal increase in the number of cells in the neuropil and rare perivascular cuffs. (H&E ×100.)

Oligonucleotide 313 (5′-GCTAGTCGACCCGTCGTAGTG-TCTGGGGATC-3′) was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Oligonucleotide 312 (5′-TAC-TGCTCGAGCCAAATGCGGCAAACCCCCCG-3′) was unlabeled. The PCR reaction contained 1× PCR buffer, 125 μM dATP, 125 μM dCTP, 125 μM dGTP, 125 μM dTTP, 0.25 nmol/ml of each oligonucleotide and 10 units/ml of Taq polymerase (Boehringer-Mannheim) and 50% by volume the product of the reverse transcriptase reaction. The reaction proceeded for 20 cycles consisting of denaturation at 94° for 1 min, annealing at 55° for 1 min, and extension for 2 min at 72°. The 168-nt reaction products were resolved by electrophoresing 5-μl aliquots from the reactions on a 6% polyacrylamide–50% urea denaturing gel and were visualized by autoradiography. Control PCR reactions contained clone P4 DNA (Vande Woude *et al.,* 1990). P4 contains the BDV sequence from B8 subcloned into pGEM 3Z.

RESULTS

Kinetics of high and low-dose-attenuated BDV infection in rats

Virulent brain-derived BDV causes an immunopathologically mediated acute encephalitis and clinical disease. Antibodies are produced in response to the infection, but high titers are achieved only after the virus has already replicated to maximal levels in neural tissues. Preliminary experiments were undertaken to examine the pathogenesis of tissue culture-attenuated BDV in weanling rats. Inoculation of attenuated BDV over a range of virus doses (10^2–10^6 $TCID_{50}$) indicated that high doses (10^5 and 10^6 $TCID_{50}$) resulted in the rapid production of antiviral antibodies, no detectable virus replication, and mild, nonprogressive encephalitis. In contrast, inoculation with attenuated BDV at low doses (10^2–10^4 $TCID_{50}$) resulted in a delay in the antibody response, productive replication of virus in the brain, and progressive encephalitis. The virological, clinical, and pathological aspects of the disease were delayed (30 days) in the rats inoculated with attenuated low-dose BDV compared to rats inoculated with virulent BDV (Carbone *et al.,* 1987).

To further investigate the kinetics of Borna disease in rats infected with attenuated BDV, a longitudinal study was undertaken in weanling rats to examine virus replication, immune responses, and the development of pathological lesions. Fourteen rats were inoculated with 10^2 $TCID_{50}$ and 23 with 10^6 $TCID_{50}$ of attenuated BDV. Rats from each of the two groups were examined at different time points after inoculation, as indicated in Table 1. Animals were examined daily for evidence of clinical disease. After sacrifice, the brain of each animal was divided in half; one half was used for virus titration and RNA isolation and the other half was fixed in formalin for histology, *in situ* hybridization, and immunohistochemistry. Blood was collected from all animals for determination of antibody levels.

The brains of the low-dose animals were negative for histopathological changes until Day 20 (Table 1), but after Day 30, most animals had developed infectivity titers of 10^4 to 10^6 $TCID_{50}$/ml. These animals had not produced detectable antibodies or inflammatory cell infiltrates to BDV by 47 days p.i., despite large amounts of virus in the brain. After this time, several of these low-dose-inoculated animals developed clinical disease and many had severe pathological lesions (Fig. 1a). Viral RNA and anti-

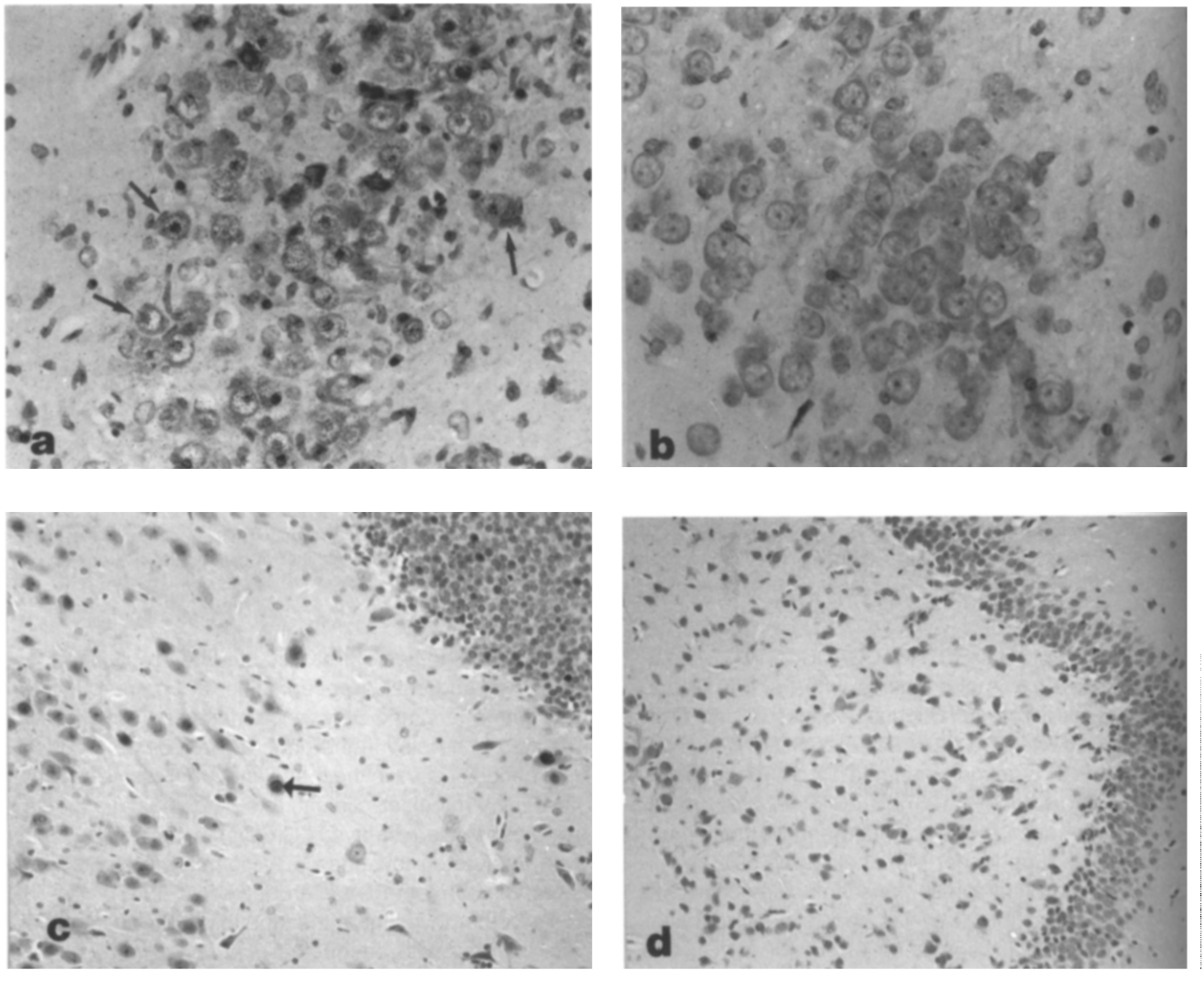

FIG. 2. Sections of brain tissue (hippocampus) from rats inoculated with 10^3 (a, c) or 10^6 (b, d) $TCID_{50}$ BDV and stained for vRNA by *in situ* hybridization (a, b) or for viral antigen by immunohistochemistry (c, d). The animals inoculated with the lower dose of virus had abundant viral RNA and antigen (arrows), whereas those inoculated with the higher dose of BDV had no evidence of viral RNA or antigen.

gen were present in large amounts in neurons and astrocytes in the cerebral hemispheres, especially in the hippocampus (Figs. 2a and 2c) and the frontal cortex of the brain. Lectin histochemical stains of brain tissue demonstrated abundant activated macrophages in the brain (Fig. 3a). There were also numerous activated astrocytes as determined by immunohistochemical staining of brain tissue with antibodies to glial fibrillary acidic protein (Fig. 3c). Northern blot analysis of RNA extracted from brain confirmed high levels of viral mRNA (Fig. 4).

None of the animals inoculated with high doses of attenuated virus had become ill by Day 68. Beyond Day 44 a few of the animals had mild encephalitis (Fig. 1b; Table 1), but most had no histological changes in the brain. No virus could be cultured from the brains of any of these animals (Table 1). Examination of tissues from these animals by *in situ* hybridization and immunocytochemistry showed no viral RNA or antigen (Figs. 2b and 2d). In contrast, BDV-specific antibodies were present in serum of high-dose-inoculated rats from Day 7 onward. Antibody titers greater than 1:1000 were detectable by Day 11 after infection. These high dose-inoculated animals had minimal macrophage infiltrates and minimal increases in glial fibrillary acidic protein expression in astrocytes when compared to the animals inoculated with the low dose of virus (Figs. 3b and 3d). Thus, animals inoculated with a high dose of BDV had minimal changes in the brain by a number of parameters.

Northern analysis of RNA extracted from brain of high-dose-inoculated animals and hybridized with BDV-specific probes was negative (data not shown). A more sensitive assay of BDV RNA using reverse transcription/PCR analysis of RNA isolated from brain showed very low but detectable levels of viral RNA (Fig. 5). This level of RNA

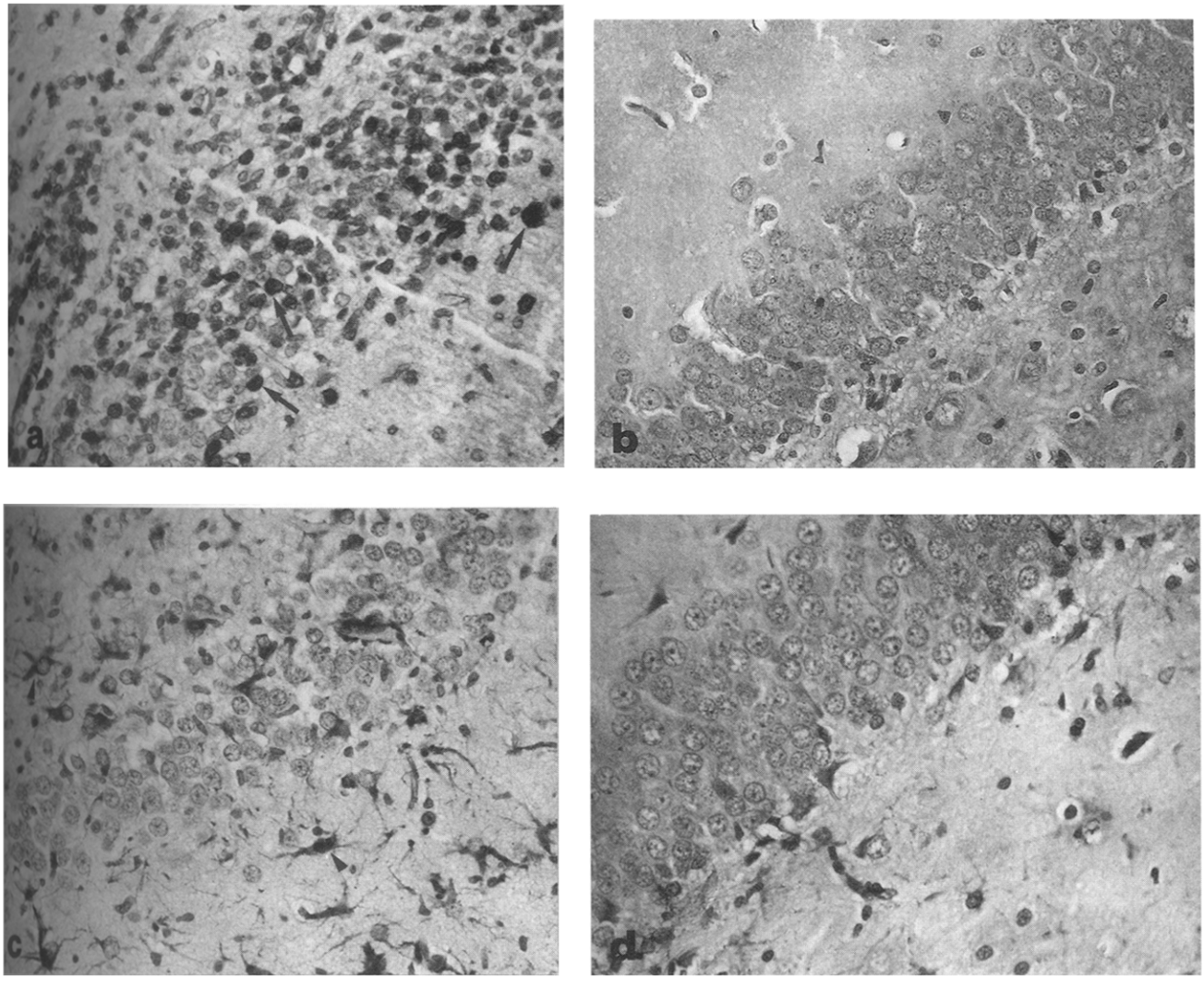

FIG. 3. Sections of hippocampus from rats inoculated with 10^3 (a, c) or 10^6 (b, d) $TCID_{50}$ BDV and stained histochemically using the lectin RCA-1 that identifies brain macrophages (a, b) and GFAP that detects astrocytes (c, d). Tissue from animals inoculated with the low-dose virus showed an influx of activated macrophages (arrows) in the neuronal layer and abundant activated astrocytes (arrowheads) in contrast to animals inoculated with the high dose of virus. (Hematoxylin ×400.)

was estimated to be 10,000-fold less than that found in brains of animals with clinical BD.

Despite the lack of infectious virus and the extremely low levels of viral RNA in the brain of the high-dose animals, inoculation of rats with 10% homogenate of brain (obtained from 12 high dose rats 47 days postinoculation) resulted in classical BD with severe encephalitis, indicating the presence of virulent virus in the brain homogenate. High titers of virus, viral RNA, and viral proteins were easily detected in brains of these recipient animals (data not shown). This provides intriguing data that low levels of BDV persisted in the brain of these rats.

Low-dose BDV/MDCK challenge of passively immunized rats

The observation that animals inoculated with a high dose of attenuated virus rapidly developed high anti-BDV antibody levels and did not develop disease suggested that the presence of antibody early in the infection might be protective. Although earlier studies failed to show a protective effect of antisera (Carbone *et al.*, 1987; Narayan *et al.*, 1983b), we tested this concept again by passively immunizing rats with sera obtained from high dose animals (anti-BDV antibody titers >1:1000) and challenging with low-dose BDV/MDCK virus. Four rats were inoculated intraperitoneally with antiserum 1 day prior to and on the day of virus challenge, and the antibody treatments were repeated on Days 7, 14, and 21 postinoculation. These four animals and four control rats were inoculated IC with 10^2 $TCID_{50}$ of attenuated BDV. On Day 7 postinfection, all rats had BDV antibody titers ranging from 1:10 to 1:100. The experiment was terminated on Day 50 and brains were examined for encephalitis and virus load. All four control animals and three of the four

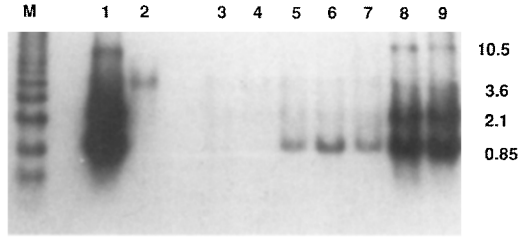

FIG. 4. Northern analysis of RNA from rats infected with low-dose infectious BDV-MDCK. Rats were inoculated with 10^2 $TCID_{50}$ of BDV-MDCK and sacrificed at various times postinfection for analysis of BDV RNA species in their brains. RNA (10 μg) was used for each sample. After electrophoresis in an agarose–formaldehyde gel and transfer to a Hybond-N nylon membrane (Amersham), BDV RNA species were detected by hybridization using an oligonucleotide probe (247) that detects BDV positive-strand RNAs. This probe shows some cross-reaction with 28S ribosomal RNA as can be seen in lane 2 (uninfected rat brain RNA), especially after the long exposure used here to enhance detection of BDV RNA species at early time points. Lanes: M, marker lane containing the end-labeled 1-kb DNA ladder (Life Sciences); 1, RNA from rat brain infected with nonattenuated rat brain-passaged BDV; 2, RNA from uninfected rat brain. Lanes 3–9 contain RNA from the brains of BDV-MDCK animals sacrificed at different times after infection: 3, 40 days p.i.; 4, 30 days p.i.; 5, 30 days p.i.; 6, 40 days p.i.; 7, 44 days p.i.; 8, 53 days p.i.; 9, 53 days p.i.

animals receiving passive immunization had equivalent amounts of virus in the brain. Encephalitic lesions were indistinguishable between the test and control groups (data not shown). Thus, passive immunization with sera failed to protect animals against virus replication or the development of lesions in the brain.

Virulent BDV challenge of rats previously inoculated with high- or low-dose BDV/MDCK virus

Since animals inoculated with high-dose-attenuated BDV had only minimal infectious virus in the brain, we

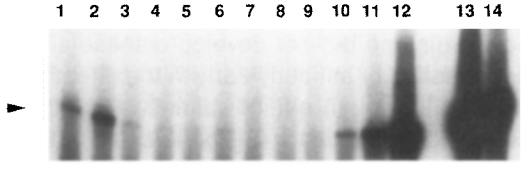

FIG. 5. Semiquantitative RT-PCR of RNA from rats infected with high-dose infectious BDV-MDCK. Rats were inoculated with 10^6 $TCID_{50}$ of BDV-MDCK and sacrificed at various times after infection for analysis of BDV RNA species present in their brains. Lanes 1–6 contain PCR products from animals infected with BDV-MDCK and sacrificed at different times postinfection: lane 1, 7 days p.i.; lane 2, 11 days p.i.; lane 3, 44 days p.i.; lane 4, 53 days p.i.; lane 5, 68 days p.i.; lane 6, 68 days p.i.; lane 7, no RNA; lane 8, RNA from uninfected rat brain. Lanes 9–12 contain RNA from rat brain infected with nonattenuated rat brain passaged BDV which has been serially diluted with RNA from uninfected rat brain. The dilutions used were: lane 9, 10^4 dilution; lane 10, 10^3 dilution; lane 11, 10^2 dilution; lane 12, 10-fold dilution. Lanes 13 and 14, control PCR reactions containing 5 pg (lane 13) and 1 pg (lane 14) of clone P4 DNA.

TABLE 2

RAT BRAIN VIRUS CHALLENGE 30 DAYS AFTER INOCULATION WITH LOW- OR HIGH-DOSE-ATTENUATED BDV[a]

Test regimen	No. of rats	Clinical disease	Pathology[b]	Virus in brain
High dose	5	0/5	N	0/5
Low dose	4	2/4	+++	4/4
Control	2	2/2	+++	2/2

[a] Rats were inoculated IC with a high (10^6 $TCID_{50}$) or low (10^2 $TCID_{50}$) dose of attenuated BDV, followed 30 days later by challenge with 10^4 $TCID_{50}$ of virulent rat brain virus. They were monitored for the development of clinical disease and sacrificed after 23 days.

[b] Severity of lesions: N, none or mild inflammation; +++, severe inflammation.

next determined whether such animals could be challenged with virulent rat brain BDV. Five rats were inoculated with 10^6 $TCID_{50}$ of attenuated BDV, four with 10^2 $TCID_{50}$-attenuated BDV, and two were sham inoculated. All 11 rats were challenged IC with 50 μl of undiluted rat brain virus (10^4 $TCID_{50}$) 30 days later. The control animals and two of the four animals inoculated with low doses of the attenuated virus developed clinical Borna disease during the following 3 weeks (Table 2). Both controls and all four low-dose animals had severe encephalitis. In striking contrast, the five animals that received high doses of the attenuated BDV remained completely refractory to challenge with the virulent virus when examined 23 days later. No trace of viral RNA or antigen was detectable by *in situ* hybridization or immunocytochemistry and no infectious virus was recovered from the brains of these animals, as assayed by inoculation of fetal rabbit glial cultures. Mild encephalitis, markedly less dramatic than that seen in the other two groups was, however, present in all five of the high-dose-inoculated animals. This pathologic response was indistinguishable from that seen in other animals that had received the high-dose virus.

DISCUSSION

In this study, intracerebral inoculation of rats with a high dose of tissue culture-attenuated BDV resulted in a rapid and vigorous anti-BDV antibody response with minimal virus replication in the brain and without the development of the immunopathologic lesions typical of Borna disease. Virus persisted in the brains of inoculated rats for at least 10 weeks without evidence of replication. When these animals were challenged with highly virulent virus from rat brain homogenate, they neither became infected nor showed signs of disease. Resistance to challenge was measured in terms of restriction of virus replication, failure to develop progressive lesions, and failure to develop clinical Borna disease. This lack of

virus replication and the resistance to virulent virus challenge correlated with the rapid and vigorous antibody response.

Our hypothesis is that these animals mounted a strong and rapid immune response to the high dose BDV. The rapid development of antibodies to BDV probably reflects the early response of both the cellular and humoral components of the immune system. The induction of an early strong cell-mediated immune (CMI) response before viral replication had progressed to exponential phase may have aborted further virus replication. These animals maintained high levels of antibodies, but minimal viral antigen was available to attract potentially immunopathologic cells to the brain and thus there was no clinical disease and only minimal pathological changes. In the case of the low-dose animals, the immune response was delayed, and virus replication proceeded leading to production of the immune response. In this case the destruction of infected cells resulted in the severe pathology typical in BD. The resistance of the high dose rats to challenge with virulent rat brain virus demonstrates that the protective immune response had been established by the high-dose infection, and not by the low-dose infection. The immune systems of the high-dose animals were able to rapidly respond to the challenge dose before the virus infected many cells. In contrast, the low-dose animals rapidly succumbed to the virulent virus, suggesting the immune response was unable to protect against challenge.

Earlier studies on BDV pathogenesis had showed that the development of lesions came on the heels of productive virus replication in brain, irrespective of the length of time the virus required to go into exponential replication. Additional studies supported this paradigm of sequential progression of Borna disease which starts with viral replication followed by CMI responses and then pathological changes. In these experiments, lesions in the brain and clinical disease developed in immunosuppressed animals within a few days following adoptive transfer of virus-specific immune cells (Narayan, *et al.*, 1983b).

The rapid decline in virus burden in the brain of high-dose-inoculated rats may be due to elimination of the few cells actively replicating virus by the putative CMI response in these animals. A similar decline in virus burden had been observed in animals surviving the necrotizing encephalitic process caused by infection with rat brain-derived virus (Carbone *et al.*, 1987). The extensive loss of neuropil in these animals is compatible with elimination of infected cells from the brain.

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