Kinetics of Virus Spread and Changes in Levels of Several Cytokine mRNAs in the Brain after Intranasal Infection of Rats with Borna Disease Virus

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We have used the reverse transcriptase-polymerase chain reaction technique to gain insight into the pathogenesis of encephalitis caused by Borna disease virus (BDV). RNA specific for BDV was first detected in the olfactory bulb of intranasally infected rats at 6 days postinfection (p.i.). At 14 days p.i., high levels of BDV RNA were found in all brain regions, and at 26 days p.i., BDV-specific RNA was also present in the eye, nasal mucosa, and facial skin. In the chronic phase of the disease, BDV RNA was identified in many peripheral organs but not in blood. Analysis of brain tissue for the presence of cytokine mRNAs revealed that the mRNA levels of interleukin-6 (IL-6), tumor necrosis factor alpha, and IL-1α had increased sharply at 14 and 26 days p.i. These cytokine mRNAs reached maximum levels at the peak of inflammatory reactions and decreased drastically in the chronic phase of the disease. Although IL-2 mRNA was also found in normal brain, it was markedly increased in BDV-infected brain at 14 days p.i. Expression of gamma interferon (IFN-γ) mRNA, which was not observed in normal rat brain, was detected at 14 days p.i. and reached a maximum level at 38 days p.i. IL-2 and IFN-γ mRNA expression correlated with expression of CD4 and CD8 mRNAs, indicating that both CD4+ and CD8+ T lymphocytes are induced in the early stages of BDV infection. Since IFN-γ and CD8 mRNA levels were still highly elevated in the chronic phase of Borna disease, it is likely that CD8+ T lymphocytes act to reduce inflammation and to ameliorate neurological signs during the chronic phase of infection.

Neurological diseases caused by conventional viruses such as lymphocytic choriomeningitis virus (1, 7), measles virus (40), rubella virus (40), papovavirus (40), and human immunodeficiency virus type 1 (12, 28) are often characterized by a prolonged asymptomatic period, evidence of immune system recognition, and the presence of inflammatory components (e.g., mononuclear inflammation) among the neuropathological changes (40). The mechanisms by which these viruses cause neurological disease are not fully understood. In many cases the virus is probably not directly involved in the destruction of brain tissue but may cause damage indirectly by triggering cell-mediated immune responses, such as activating cytotoxic T cells and macrophages. Activated macrophages secrete cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α), which can cause toxic effects in the brain (29). Additionally, other soluble factors, such as neurotoxins (10), quinolinic acid (14), and excitatory neurotransmitters (38), have been implicated as toxin candidates responsible for neurological damage.

Borna disease (BD) in rats is an animal model that could provide insight into the pathogenesis of virus-mediated neurologic disorders for the following reasons: (i) BD virus (BDV), like human immunodeficiency virus type 1, is an RNA virus that can have a long latent period between infection and clinical manifestations (20); (ii) BDV has a specific affinity for neurons in the limbic system and can produce an observable neurobehavioral syndrome in rats (26); and (iii) BD is a cell-mediated disease of the brain of immunocompetent animals (26, 30, 31).

Although BD is endemic only in horses and sheep and occurs spontaneously only in some parts of Germany and Switzerland, it has a broad host range extending from birds to primates (20). On the basis of serologic data, BDV may also exist in humans, where it has been associated with certain behavioral disorders (32, 39).

BD has been most extensively studied in rats (2, 26), in which the outcome of the infection depends on the age of the animals. Infection of newborn rats results in a persistent infection. Despite a massive virus load in the brain, these rats do not develop any inflammatory cell infiltrates in the central nervous system (CNS) and do not show severe signs of neurological disease (15, 26). However, persistently infected rats express significant learning deficiencies together with subtle behavioral alterations (6, 25). In immunocompetent adult rats, the disease is biphasic, beginning with a transient necrotizing encephalitis. In this state of the disease, the animals show hyperactivity, aggressiveness, and ataxia (2, 15, 26). This acute phase lasts from 2 to 3 weeks and is followed by a chronic phase in which the animals become listless or obese (2, 15, 26). BDV, the putative agent of BD, is unclassified, and its morphological characterization has so far remained elusive. Recently, the BDV genome has been partially cloned by using subtraction cDNA libraries prepared either from BDV-infected and uninfected rat brain (19) or from BDV-infected and uninfected tissue culture cells (39). It has been suggested that the BDV genome is an RNA (4, 19, 39).

We used the reverse transcriptase-polymerase chain reaction (RT-PCR) method to analyze the tissue distribution of BDV-specific RNA during different stages of infection. In addition, we examined virus-induced changes in brain mRNA levels of several macrophage and lymphocyte-de-

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rived cytokines and the T-lymphocyte markers CD4 and CD8 and compared these changes with the BDV RNA expression and histopathological alterations observed in the brains of acutely and chronically BDV-infected rats.

MATERIALS AND METHODS

Preparation and titration of virus. BDV was kindly provided by W. Herbst, University of Giessen, Giessen, Germany. The virus was grown in rabbit kidney cells (RK13). Infected cells were scraped from tissue culture flasks and centrifuged. The pelleted cells were removed, and 0.5 ml of them was suspended in 4.5 ml of Dulbecco's minimal essential medium plus 10% fetal calf serum and sonicated for 5 min at 4°C. The cell homogenate was then clarified by centrifugation for 10 min at 1,200 × g at 4°C and stored at −70°C.

The virus preparation was titrated by using BHK 21 cells grown on round coverslips in 24-well culture dishes. The culture medium was Dulbecco's minimal essential medium plus 10% fetal calf serum. After the cells had reached about 80% confluence, the tissue culture supernatant was removed, 100 μl of serial dilutions of the virus preparation was added per well, and the cells were incubated for 24 h at 37°C. Then the virus inoculum was removed, and the cells were replenished with 200 μl of Dulbecco's minimal essential medium plus 10% fetal calf serum per well and incubated for 4 days at 37°C. The cells were then fixed, and the infectivity was detected by indirect immunofluorescence as described elsewhere (15). Virus titers were calculated from the number of fluorescent focal units (FFU) resulting from infection with the highest virus dilution.

Animals. Six-week-old female Lewis rats were used throughout the study. The animals were anesthetized with methoxyflurane (Metofane; Pitman-Moore Inc., Mundelen, Ill.) and infected intranasally in each nostril with 30 μl containing 3 × 10^4 FFU of BDV. At different times postinfection (p.i.), two rats were killed at each time point by methoxyflurane inhalation and the brain, spinal cord, and various nonneuronal tissues were collected. The brain was dissected into the bulbous olfactorius, cerebrum, brain stem, and cerebellum.

RNA extraction, reverse transcription, and amplification of cDNA. All tissue samples were immediately placed in 2 ml of 4 M guanidine isothiocyanate containing 50 mM Tris-HCl (pH 7.2), 10 mM EDTA, and 1% 2-mercaptoethanol. The RNA was extracted by the standard guanidine thiocyanate-CsCl gradient centrifugation method, as described previously (33). The RNA (1 μg) was incubated in 20 μl of 50 mM Tris-HCl (pH 8.3)–75 mM KCl–10 mM dithiothreitol–3 mM $MgCl_2$–0.5 mM (each) deoxynucleoside triphosphate (dNTP)–1 μM primer–20 U of RNasin (Promega)–200 U of Moloney murine leukemia virus RT (Bethesda Research Laboratories, Bethesda, Md.) for 1 h at 42°C. Either a portion or all of the reverse transcription products were subsequently subjected to PCR. The PCR amplification was done in 100 μl of 10 mM Tris-HCl (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–0.01% (wt/vol) gelatin–200 μM (each) dNTP–1 μM (each) primer–2.5 U of Amplitaq polymerase (Perkin-Elmer Cetus). The reaction mixture was overlaid with 100 μl of mineral oil, and the PCR was performed in a thermal cycler (Perkin-Elmer Cetus) for 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 90 s, and polymerization at 72°C for 3 min, followed by a final polymerization step of 10 min at 72°C. After the PCR, 100 μl of chloroform was added to recover the aqueous phase for further analysis.

Oligomers, both upstream and downstream of the 38-kDa protein gene of BDV, were employed to amplify a 546-bp segment of the gene. The nucleotide sequences of the oligonucleotide primers used for RT-PCR and the hybridization probe are as follows: Borna antisense 26-mer, 5' GGG TAGCATCCATACATTCTGCGAGG 3'; Borna sense 24-mer, 5' CAGTAACGCCCAGCCTTGTGTTTC 3'; and hybridization probe 22-mer, 5' AACGGCCAGCCCTGGGT AGGCT 3' (18a). These primers were also used to prime selectively either the positive-strand viral mRNA or the negative-strand viral genomic RNA in the RT reaction. An aliquot of the PCR products was analyzed on a composite gel containing 3% NuSieve agarose and 1% SeaKem agarose (FMC Bioproducts, Rockland, Maine). The gels were blotted onto GeneScreen nylon membranes (DuPont, Boston, Mass.), hybridized to ^{32}P-labeled specific oligomer, and exposed to Kodak X-Omat AR film at −80°C. The RT-PCR amplifications and analysis for the mRNAs of rat cytokines IL-1α, IL-2, IL-6, TNF-α, and alpha and gamma interferon (INF-α and INF-γ) and the rat T-lymphocyte markers CD4 (W3/25 antigen) and CD8 (37,000-molecular-weight [37K] chain of the CD8 antigen) were performed with oligomers to amplify segments of 589, 504, 636, 288, 468, 413, 621, and 546 bp, respectively. Total rat cerebrum RNA (8 μg) and 1 μM antisense primer were used in the RT reaction. The nucleotide sequences of the oligonucleotide primers used in these RT-PCR reactions are as follows: IL-1α antisense 22-mer, 5' CTTTATCCTACCCATCCG GCAC 3'; IL-1α sense 22-mer, 5' CACAGGTAGTGAGAC CGACCTC 3'; IL-2 antisense 23-mer, 5' GAGCCCTTGGG GCTTACAAAAAG 3'; IL-2 sense 22-mer, 5' CAGGTGC TCCTGAGAGGGATCG 3'; IL-6 antisense 31-mer, 5' CTAGGTTTGCCGAGTAGACCTCATAGTGACC 3'; IL-6 sense 34-mer, 5' ATGAAG TTTCTCTCCGCAAGAGACT TCCAGCCAG 3'; TNF-α antisense 32-mer, 5' CTACG ACGTGGGCTACGGGCTTGTCACTCGAG 3'; TNF-α sense 31-mer, 5' ATGAGCACGGAAAGCATGATCCGAG ATGTGG 3'; IFN-α antisense 30-mer, 5' GGCTGAGGAA GACAGGGCTCTCCAGACTTC 3'; IFN-α sense 30-mer, 5' TGTGACCTGCCTCATACTCATAACCTCAGG 3'; IFN-γ antisense 32-mer, 5' TCAGCACCGACTCCTTTTCCGCT TCCTTAGGC 3'; IFN-γ sense 30-mer, 5' GTTACTGCC AAGGCACACTCATTGAAAGCC 3'; CD4 antisense 34-mer, 5' CTTGGGTGAGGTGGGTCCCATCACCTCACA GGTC 3'; CD4 sense 34-mer, 5' CCGGGTACCAGACTGT TGCAGGGGCAGAGCCTGA 3'; CD8 antisense 28-mer, 5' CATGAAGTGAATCCGGGCTCTCCTCCGC 3'; CD8 sense 31-mer, 5' CTCCTTCAGACTCCTTCATCCCTGC TGGTTC 3'; rat β-actin antisense 21-mer, 5' GTGTGGTGC CAAATCTTCTCC 3'; rat β-actin sense 20-mer, 5' GCGC TCGTCGTCGACAACGG3'. The nucleotide sequences of the probes are as follows: IL-1α 48-mer, 5' GTAAGAGAA GAGCAAAGCCTAGTGGAACCAGCCCGACATATGAT ACTG 3'; IL-2 50-mer, 5' GCCAATTCGATGATGAGCCA GCAACTGTGGTGGAATTTCTGAGGAGATGG 3'; IL-6 49-mer, 5' GGTCTGTTGTGGGTGGTATCCTCTGTGAAG TCTCCTCTCCGGACTTGTG 3'; TNF-α 40-mer, 5' CTTC TCATTCCTGCTCGTGGCGGGGGCCACCACGCTCTTC 3'; IFN-α 50-mer, 5' GCAGCAGGTAGGGGTGCAGGAAT CTCCCCTGACCCAGGAAGACTCCCTAC 3'; IFN-γ 48-mer, 5' GACAACCAGGCCATCAGCAACAACATAAG TGTCATCGAATCGCACCTG 3'; CD4 47-mer, 5' GGAAG AAGGAGCCTTCTCTGCCTTCCATCTCAACTCTCCCT GCAGCG 3'; CD8 47-mer, 5' CTCCCAACCATCGCGC AGAAGTAGAAGCCACTGTCCTCTGGCTTCAC 3'; rat β-actin 30-mer, 5' GCTCCCCGGGCCGTCTTCCCCTC CATCGTG 3'.

Histopathology. Brains were fixed in 10% buffered formalin. The tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for light microscopy.

RESULTS

Appearance of clinical signs and brain lesions after intranasal infection with BDV. Six-week-old female Lewis rats were infected intranasally with 3×10^4 FFU of BDV. The animals were observed for the appearance of clinical signs, and at different time intervals, two animals were euthanized and their brains and various other tissues were collected. One half of each brain was processed for histopathology, and the other half was subjected to RT-PCR analysis. The data presented in Fig. 2 to 5 represent results obtained from tissue analysis from one rat per time interval. Tissue analysis from a second animal yielded similar results and is not presented.

During the first 20 days after infection, no clinical signs were observed in BDV-infected animals. The animals euthanized 26 days p.i., however, were exhibiting severe neurological signs such as convulsion and tremor, while the animals killed 38 and 60 days p.i. were in the passive phase of the disease, characterized by an apathetic behavior.

Rat brains collected 14 days after infection showed only mild mononuclear cell infiltrates in the meninges of the rostral cerebrum. Between 21 and 26 days after infection, a moderate multifocal perivascular cuffing and some necrosis were observed in the olfactory bulb and adjacent rostral cerebrum. There was also a mild diffuse meningeal infiltration with mononuclear cells in these areas. All other brain areas, including brain stem and cerebellum, appeared normal. Brain lesions observed 38 and 60 days after infection were similar but much milder compared with those seen 26 days p.i.

Kinetics of virus spread within the CNS and nonneuronal tissues. The tissue distribution of BDV during different stages of infection was analyzed by using RT-PCR to amplify BDV-specific RNA. An aliquot of the PCR product was analyzed on an agarose gel, and after transfer to a nylon membrane, the BDV-specific fragment was identified with a ^{32}P-labeled 38-kDa gene-specific oligomer. To determine the sensitivity of the RT-PCR assay, total RNA was isolated from rat brain homogenate containing 5×10^6 FFU of BDV and serial dilutions of the RNA were then subjected to RT-PCR. The smallest amount of BDV-specific RNA that was detected after PCR amplification was 0.6 FFU (data not shown). To determine the specificity of the RT-PCR, RNA isolated from normal rat brain and BDV-infected rat brain was selectively amplified by using sense or antisense primers. We show in Fig. 1 that BDV-specific RNA can be detected only in BDV-infected rat brain and that almost equal amounts of RNA were amplified when sense or antisense primers were used for RT reaction of BDV RNA. Amplification of β-actin mRNA served as an internal control.

The RT-PCR method was used to analyze different brain areas and various nonneuronal tissues at different times after infection for the presence of BDV RNA. As early as 6 days p.i., BDV RNA was identified, but only in the olfactory bulb (Fig. 2b). At 14 days p.i., BDV-specific RNA was detected in all brain areas and the spinal cord (not shown), and at 26 days p.i. BDV RNA expression in the brain reached its maximum level. BDV RNA expression in the subacute and chronic phases of the disease at 38 and 60 days p.i. was similar to that seen at 26 days p.i. (Fig. 2a). While at 14 days
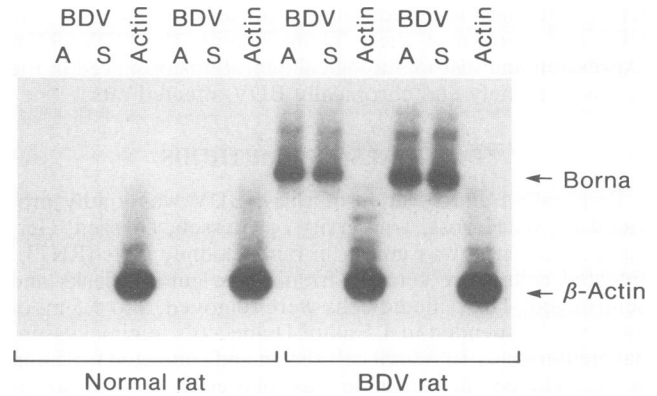

FIG. 1. Selective amplification of BDV-specific positive- and negative-strand RNA. Total RNA (1 μg) isolated from the brains of normal and BDV-infected rats was reverse transcribed with either an antisense (A) or a sense (S) primer. The reverse transcription products were then subjected to PCR amplification and analyzed as described in Materials and Methods. Amplification of a 248-bp β-actin mRNA served as an internal control.

p.i., BDV RNA was detected only in the brain, at 26 days p.i. BDV RNA was also present in the eye, nasal mucosa, and facial skin (Fig. 3). In the subacute and chronic phases of the disease (Fig. 3, day 38 and day 60), the virus had invaded many other nonneuronal tissues, including the salivary glands (lanes d and e), stomach (lanes g), small intestine (lanes h), heart (lanes f), skeletal muscle (lanes i), adrenals (lanes j), bladder (lanes k), and ovaries (lanes l). We were unable to detect BDV RNA in the blood, lung, liver, pancreas, spleen, cervical lymph node, thymus, and cerebrospinal fluid at any stage of the disease.

Effects of BDV infection on mRNA levels of IL-1α, IL-2, IL-6, TNF-α, IFN-α, and IFN-γ. We used the RT-PCR method to examine the possibility that BDV induces several
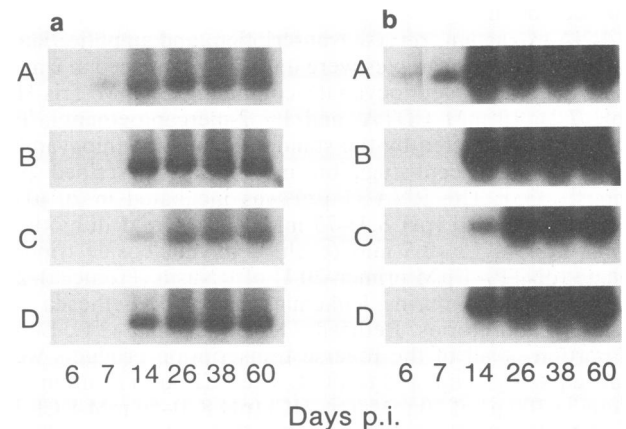

FIG. 2. Appearance of BDV-specific RNA in rat brain after intranasal inoculation of BDV. Rats were infected intranasally with 3×10^4 FFU of BDV. At different days after infection, total RNA was extracted from the bulbus olfactorius (A), cerebrum (B), cerebellum (C), and brain stem (D). The results displayed represent RT-PCR products obtained from one rat per time point. Total RNA (1 μg) was subjected to RT-PCR, and the amplified BDV-specific cDNA was analyzed as described in Materials and Methods. (a) One-hour exposure time; (b) 6-h exposure time. Animal care was in accordance with institutional guidelines.
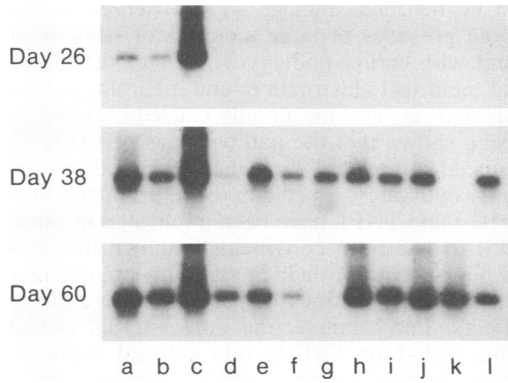
Day 26

Day 38

Day 60

a b c d e f g h i j k l

FIG. 3. Appearance of BDV-specific RNA in nonneuronal tissues after intranasal inoculation of BDV. Rats were infected intranasally with 3×10^4 FFU of virus. At different days after infection, total RNA was extracted from the nasal mucosal membrane (lanes a), facial skin (lanes b), eye (lanes c), glandular parotid (lanes d), glandular submandibularis (lanes e), heart (lanes f), stomach (lanes g), small intestine (lanes h), gastronemius muscle (lanes i), adrenals (lanes j), bladder (lanes k), and ovaries (lanes l). Total RNA (1 μg) was subjected to RT-PCR, and the amplified BDV-specific DNA was analyzed as described in Materials and Methods. The results displayed represent RT-PCR products obtained from one rat per time point.

IL-1

IL-2

IL-6

TNF-α

INF-α

INF-γ

β-Actin

NR 3 7 14 26 38 60

Days p.i.

FIG. 4. Effects of BDV infection on the levels of mRNAs of IL-1α, IL-2, IL-6, TNF-α, INF-α, and INF-γ in rat brain. Rats were infected intranasally with 3×10^4 FFU of BDV, and at intervals after infection RNA was isolated from the cerebrum. Eight micrograms of total RNA was used for each RT-PCR, and the PCR products were identified by using cytokine-specific hybridization probes as described in Materials and Methods. Amplification of β-actin mRNA, using 1 μg of total RNA in the RT reaction, served as an internal control. The results displayed represent RT-PCR products obtained from one rat per time point.

cytokines in the brain. While no or only very small quantities of IL-1α, IL-6, TNF-α, and IFN-γ mRNA were detected in uninfected rat brains, there was a sharp increase of these mRNAs at 14 days p.i. (IL-6, TNF-α, and IFN-γ) or 26 days p.i. (IL-1) (Fig. 4). Maximum levels of IL-6 mRNA and TNF-α mRNA expression were seen at 26 days p.i., coinciding with the peak of the acute phase of BD. The highest levels of IFN-γ mRNA, however, were observed in the subacute phase of the disease at 38 days p.i. In the chronic phase of the disease (60 days p.i.), the levels of mRNA for IL-6, TNF-α, and also IL-1 had decreased sharply while IFN-γ mRNA was still present at a relatively high level, comparable with that at 26 days p.i. Although significant amounts of IL-2 mRNA were detected in normal rat brain, the level of this mRNA was increased markedly beginning on day 14 p.i. Higher levels of IL-2 mRNA in the brain of the control in comparison with the levels measured from brains of the infected rats at 3 and 5 days p.i. are not likely due to variation in the total RNA used in the experiment, because the levels of actin mRNA were similar. Analysis of IL-2 mRNA from several normal rats demonstrated like patterns of variation (data not shown) compared with those from the normal control rat and the rats at 3 and 7 days p.i., as shown in Fig. 4. No differences in the expression of IFN-α mRNA were observed between normal and BDV-infected rat brain.

BDV-induced changes of the mRNA levels of CD4 and CD8 surface proteins. We used the RT-PCR method to determine the induction of CD4+ and CD8+ T lymphocytes in the brain by analyzing mRNA levels of the CD4 and CD8 surface marker proteins. Although significant amounts of CD4 mRNA were present in normal rat brains, an increase of this mRNA was seen in BDV-infected rat brains 26 and 38 days p.i. (Fig. 5). The CD8 mRNA, detectable in BDV-infected brain beginning at day 14 p.i., reached its maximum at 26 days p.i. and persisted through day 60 p.i. at an almost constant level.
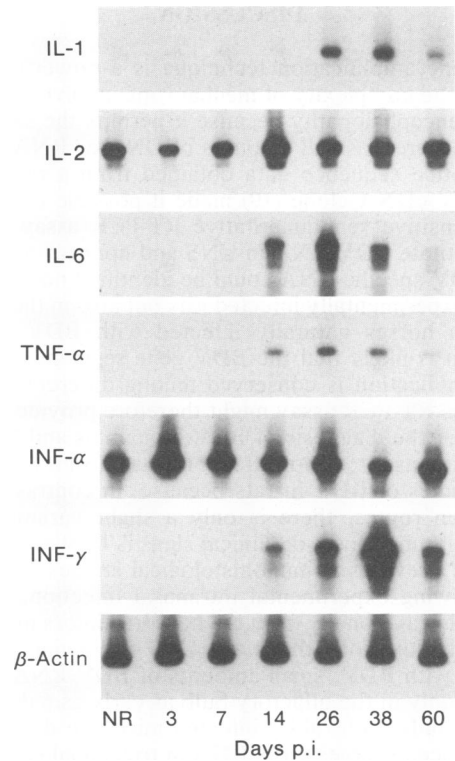
CD4
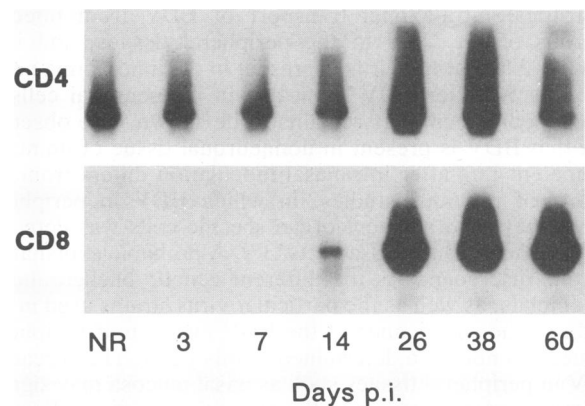
CD8

NR 3 7 14 26 38 60

Days p.i.

FIG. 5. Detection of mRNAs of the CD4 and CD8 lymphocyte surface proteins in uninfected and BDV-infected rat brain. Rats were infected intranasally with 3×10^4 FFU of BDV. On different days after infection, RNA was isolated from the cerebrum and 8 μg of total RNA was subjected to each RT-PCR. The PCR products were identified by using CD4 and CD8-specific hybridization probes as described in Materials and Methods. The results displayed represent RT-PCR products obtained from one rat per time point.

DISCUSSION

The gene amplification technique is a powerful tool for studying the complexity of mechanisms involved in a virus-induced encephalopathy because it permits the semiquantitative measure of small amounts of DNA or RNA (17, 22).

Nucleotide sequence data obtained from a recently isolated BDV cDNA clone (19) made it possible to develop a highly sensitive semiquantitative RT-PCR assay to detect and quantitate BDV RNA in CNS and nonneuronal tissues. Since BDV-specific RNA could be identified not only in the CNS of experimentally infected rats but also in the brains of European horses naturally infected with BDV (data not shown), it is likely that the BDV gene segment utilized for PCR amplification is conserved among different BDV isolates. This RT-PCR assay might therefore provide a general method for the diagnosis of BDV in animals and humans.

The intranasal route of infection was chosen for pathogenicity studies of BDV in rats because, in contrast to other inoculation routes, there is only a slight variation in the incubation time (onset of clinical signs is between 21 and 24 days) (2). Previous immunohistological studies have shown that following experimental intranasal infection, the virus migrates intra-axonally from the neuroreceptors in the olfactory epithelium into the brain (24). Following intranasal infection with BDV, small amounts of BDV RNA could be detected only in the olfactory bulb as early as 6 days p.i. In a recent study with rabies-infected mice, we demonstrated the presence of rabies virus RNA in trigeminal ganglia at 18 h after injection of rabies virus in the masseter muscle, indicating that the virus had entered the CNS directly without prior local replication (36). Our RT-PCR data with BDV-infected rats, however, did not reveal where BDV is retained during the first 5 days after infection. At 14 days p.i., BDV RNA expression had reached high levels in the olfactory bulb, cerebrum, brain stem, and cerebellum. RT-PCR analysis of tissues other than CNS tissues showed that BDV RNA was present in the eye, facial skin, and nasal mucosa at 26 days p.i., and at 38 and 60 days p.i. the virus had invaded many other organs, such as skeletal muscle, salivary glands, and the digestive tract. Because no BDV RNA was found in the blood, the presence of BDV RNA in tissues other than CNS tissues is most likely the result of centrifugal intra-axonal transport of BDV from infected neurons of the CNS to the peripheral tissues, and it is conceivable that the virus is present in the innervating nerve endings. Whether BDV replicates in nonneuronal cells of immunocompetent rats remains to be shown. The observation that BDV is present in nonneuronal tissue of immunocompetent rats after intranasal inoculation differs from the results of previous studies, in which BDV in peripheral nerve fibers and adjacent organ-specific cells was detected only in immunosuppressed rats (37). A probable explanation for this discrepancy is the different genetic background of the animals, as well as the particular virus strains used in the studies. The significance of the BDV invasion of peripheral tissues has not been determined at this point. The spread of BDV in peripheral tissues such as nasal mucosa may signify an important mechanism in the transmission of the disease. The observation that BDV RNA can be readily detected in such tissues as the skin may have practical implications for the in vivo diagnosis of BDV in animals. Furthermore, screening of skin biopsy material from mental patients by RT-PCR could prove decisively whether BDV is prevalent in humans and associated with particular mental disorders.

The observation that at 14 days p.i. only slight inflamma-tion and no neuronal damage were observed in the brain despite the presence of large amounts of BDV RNA is in agreement with earlier findings which support the assumption that neuronal destruction and neurological signs in BDV-infected rats are not directly caused by BDV (15, 26). It has been shown that the pathogenesis of BD is due to a virus-induced cell-mediated mechanism (30, 31).

Recently cytokines, especially those produced by macrophages (IL-1 and TNF), have been implicated in causing cell death (18). It has been convincingly shown that cytokines not only play a central role in modulating immune responses and inflammatory reactions but also can have direct cytotoxic effects. For example, the intracisternal challenge of recombinant IL-1 or TNF in rats induced meningitis and blood-brain barrier damage, and the two cytokines were synergistic in inducing these effects (29). These experimental data, together with findings that in several inflammatory and demyelinating diseases of the brain, such as AIDS-associated progressive encephalopathy (23) and subacute sclerosing panencephalitis (16), the TNF levels in serum or cerebrospinal fluid are considerably increased, support the notion that production of certain cytokines in the brain may contribute to neurological disease. To obtain information on the role of cytokines in the pathogenesis of BD, we have measured changes in the expression of several cytokine mRNAs in the brain during different stages of illness. This analysis clearly demonstrated that the levels of mRNAs to TNF-α and IL-6 increased sharply at 14 days p.i. and the level of mRNA to IL-1 increased sharply at 26 days p.i. Maximum levels of these cytokine mRNAs were reached 26 days p.i. at the peak of inflammatory reactions and neurological damage in the brain. In the chronic phase of the disease 60 days p.i., mRNA levels of these lymphokines decreased drastically, indicating that levels of expression of IL-1α, TNF-α, and IL-6 correlate with the degree of inflammation in the brain and severity of neurological signs.

In addition to the macrophage-derived cytokine mRNAs, increased levels of IL-2 and IFN-γ mRNA were also seen in the brain during acute and chronic phase of BD. Since expression of IL-2 and IFN-γ mRNAs correlates with mRNA levels of CD4 and CD8 during different stages of infection, it is likely that IL-2 mRNA is derived from CD4$^+$ T cells and IFN-γ mRNA is derived from CD8$^+$ T cells. Although significant amounts of IL-2 and CD4 mRNAs were seen in the brain tissue of uninfected rats, the levels of these mRNAs increased in the brains of BDV-infected rats 14 or 26 days p.i., confirming earlier findings which demonstrate that BDV antigen-specific CD4$^+$ T cells are induced relatively early in infection (5, 30). These CD4$^+$ T cells are believed to play a pivotal role in the immune pathological mechanism of BD (5, 30, 31). The relevance of CD4 and IL-2 mRNA in normal rat brain is not known. Recently the presence of CD4 mRNA and 5'-truncated CD4 mRNA in normal human brain has been established, while mRNA to CD8 was absent (8). In addition, a CD4-related mRNA has also been observed in normal adult mouse brain (11). The detection of CD8 and IFN-γ mRNAs in BDV-infected rat brain suggests that, in addition to CD4$^+$ T-helper cells, CD8$^+$ cytotoxic T cells are also induced in early stages of infection. Previous studies reported only low levels of CD8$^+$ T cells in perivascular infiltrates and the neuropil of BDV-infected rat brains, and it has been suggested that CD8$^+$ T cells do not play a relevant role in BD (5). However, our results which demonstrate elevated levels of CD8$^+$ and IFN-γ mRNAs during the chronic phase of infection indicate that cytotoxic T cells may actually play an important role not only in inducing acute

encephalitis but also in the process of recovery from the acute phase of infection. Because of their lytic activity, $CD8^+$ T cells could be responsible for tissue necrosis and neuronal loss and additionally, through IFN-γ production, could induce major histocompatibility complex expression on astrocytes and microglia cells (9), thereby enhancing and perpetuating $CD4^+$ T-cell responses in the brain. One of the unique features of BD is the high recovery rate from acute encephalitis (2, 15, 26). It is possible that cytotoxic $CD8^+$ T cells contribute significantly to the recovery process by producing IFN-γ or other soluble factors that exert antiviral activity (34). It has been previously shown that after adoptive transfer of antiviral H-2-restricted cytotoxic lymphocytes, viral materials are cleared, in the absence of lysis, from the CNS of mice persistently infected with lymphocytic choriomeningitis virus, and it has been suggested that soluble factors released by these cytotoxic T cells are responsible for the immune clearance of products from the CNS (27). However, in the case of BDV infection, large amounts of BDV RNA and high virus titers (15, 26) were found in the brains of chronically infected rats, and therefore virus clearance does not appear to be responsible for the progression from acute to chronic BDV infection. The reduction of inflammation and amelioration of clinical signs in the chronic phase of BD could rather be due to the production of certain cytokines such as IFN-γ, which have the effect of protecting against neuronal damage. It has been shown that IFN-γ has a strong synergistic effect in the TNF- and IL-1-mediated induction of manganese superoxide dismutase, a mechanism which has been implicated in the protection of healthy cells from toxicity of O_2^- during an immune response (13). It has also been suggested that the self-limiting effect of the disease at later stages of BDV infection may be due to the action of T suppressor cells (25).

One of the remaining open questions in BDV-induced encephalitis is how inflammatory reactions are initiated in the CNS. Recently it has been demonstrated that BDV can replicate in astrocytes (3) and therefore could possibly induce direct major histocompatibility complex expression in these cells by an IFN-γ-independent mechanism (21). However, astrocytes, even when expressing the major histocompatibility complex, are unable to stimulate unprimed $CD4^+$ and $CD8^+$ lymphocytes (35). Since there is no evidence that any resident cells in the CNS can prime a T-cell response, it is concluded that CNS inflammation cannot occur before peripheral sensation (35). Although we cannot exclude the possibility of a primary replication in the olfactory epithelium at the site of inoculation, our PCR data on tissue distribution of BDV indicate that the virus replicates in the early phase of infection only in the CNS. Therefore, it is difficult to envisage how a peripheral sensation can occur. In order to answer the critical question of how CNS inflammation is triggered in BD, future efforts must focus on the very early events in BDV infection.

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