Persistent Borna disease virus infection of the brain can be prevented by treatment of naive rats with a virus-specific CD4+ T-cell line prior to infection. In rats receiving this treatment, only a transient low-level encephalitis was seen compared to an increasingly inflammatory reaction in untreated infected control rats. Virus replication was found in the brain for several days after infection before the virus was cleared from the central nervous system. The loss of infectivity from the brain was confirmed by negative results by reverse transcription-PCR with primers for mRNA, by in situ hybridization for both genomic and mRNA, and by immunohistology. Most importantly, in vitro assays revealed that the T-cell line used for transfusion had no cytotoxic capacity. The kinetics of virus clearance were paralleled by the appearance of CD8+ T cells and the expression of perforin in the brain. Testing of lymphocytes isolated from the brains of CD4+ T-cell-treated rats after challenge revealed high cytotoxic activity due to the presence of CD8+ cytotoxic T cells at time points when brain lymphocytes from infected control rats induced low-level cytolysis of target cells. Neutralizing antiviral antibodies and gamma interferon were shown not to be involved in the elimination of virus from the brain.

Borna disease (BD) is a naturally occurring or experimentally induced encephalitis caused by infection with BD virus (BDV), a single-stranded RNA virus with a remarkably wide host spectrum (25). Recent data suggest that BDV can infect humans and might be related to psychiatric disease (6, 9, 10, 26, 27). In experimentally infected rats (14, 18, 32) as well as diseased horses (3), BD is based on an immunopathological reaction in the brain. Both CD4+ and CD8+ T cells have been found in the brains of infected rats and ungulates and participate in the inflammatory response (3, 11, 24, 29). However, their effects on the development and consequences of the encephalitic reaction appear to be quite distinct. There is an increasing body of evidence that CD4+ T cells act as T helper cells, whereas CD8+ T cells exert effector functions by destroying virus-infected cells leading to a severe degenerative disease of the brain (reviewed in references 5 and 29). The presence of CD8+ T cells in vivo and the detection of major histocompatibility complex (MHC) class I-restricted cytotoxicity in brain lymphocyte preparations in vitro could be correlated with the presence of MHC class I antigen in the brain, the onset of disease and, finally, the appearance of cellular degeneration of brain cells, including virus-infected neurons (4, 8, 20, 21, 28). The important role of cytotoxic lymphocytes in cytodestructive mechanisms resulting in massive degeneration of brain cells was demonstrated by adoptive transfers of lymphocytes isolated from the brains of diseased rats. Transfer of brain lymphocytes caused an early onset of disease in infected recipients, as represented by severe neurological symptoms and a marked spongiform degeneration with premature cortical brain atrophy (28). In the same study, besides an exceedingly high cytotoxic activity exerted by CD8+ T cells, we demonstrated the entry of cells from perivascular areas into the brain parenchyma after adoptive transfer. As for the role of CD4+ T cells in BD, so far we have found no evidence that this T-cell population directly participates in brain tissue destruction; e.g., we found no evidence for MHC class II-restricted cytotoxic activity in isolated brain lymphocytes or virus-specific CD4+ T-cell lines (20, 21, 28). Furthermore, the distribution pattern of CD4+ and CD8+ T cells supports an effector role for CD8+ T cells; the latter are found predominantly in the brain parenchyma, whereas the vast majority of CD4+ T cells accumulate perivascularly (4, 20). Despite the vigorous local cellular immune response in the brain, the virus is not eliminated from the host. An explanation for this finding might be that the immune response is induced and/or recruited too slowly to the brain. Passive immunization with a BDV-specific CD4+ T-cell line was shown by Richt et al. to inhibit virus replication, and rats were protected from immune-mediated disease (23). This particular virus-specific CD4+ T cell exhibited MHC class II-restricted lysis in vitro, but the CD4 T-cell-mediated effects on virus elimination were not analyzed further. Here, we report on experiments with a noncytolytic BDV-specific CD4+ T-cell line that is able to confer protection against BDV infection and disease by enhancing the activity of virus-specific CD8+ T cells in the brain.

MATERIALS AND METHODS

Virus and experimental animals. Giessen strain He/80 of BDV was used for this study. Female Lewis rats were infected at the age of 5 weeks by injection into the left hemisphere with 5 × 105 50% tissue culture infective doses (TCID50) of BDV.

Clinical evaluation. All experimental animals were examined daily and weighed, and disease symptoms were scored by two independent observers on an arbitrary scale from 0 to 3, based on the rats’ general state of health (0.25 to 0.5, ruffled fur and hunchback) and the appearance of neurologic symptoms (1, slight incoordination and fearfulness; 2, distinct ataxia or slight paresis; 3, paresis or paralysis). The percentage of change from weight at the day of infection (100%) was calculated.
Infecitivity assay and antigen detection. Assays were done essentially as described before (30). Briefly, virus infectivity from brain homogenates was determined on rabbit embryo brain indicator cells by immunocytochemical staining with rat hyperimmune sera or BDV-specific monoclonal antibody (MAb) (33) and an antiviral peroxidase conjugate. The reaction was visualized by the addition of amino-9-ethylcarbazol. The detection limit of this assay is 10 TCID50 (1 log2).

Antibody titration and neutralization assay. All sera were tested in twofold dilution in a solid-phase enzyme-linked immunosorbent assay (ELISA) with a purified antigen from BDV-infected rat brains containing the most abundant BDV-specific proteins, namely, p40 (nucleoprotein) and p24 (phosphoprotein) (32, 33). Virus neutralization was performed essentially as described previously (12). Briefly, 50 TCID50 of BDV were incubated with serial twofold dilutions of heat-inactivated serum (at 56°C for 30 min) for 1 h. The reaction mixture was added to rabbit embryonal brain cells and incubated for 6 days. The dilution of serum required to reduce the TCID50 by 50% was defined as the neutralization titer (NT50). In all assays, titers of a serum pool from rats infected for 15 weeks or longer (12) were set as an NT50 of ≥1:1,024.

Propagation of the T-cell lines. The induction and propagation of T-cell lines were done essentially as reported before (21). Briefly, 10- to 10-week-old Lewis rats were immunized in both hind footpads with virus-specific antigen containing p24 and p40 which were purified from BDV-infected rat brain by affinity chromatography. Ten to 12 days later, the animals were anesthetized and the popliteal lymph nodes were removed. Lymphocytes were separated by Ficoll-Hypaque (Cedarlane, Hornsby, Canada) gradient centrifugation. In a secondary in vitro restimulation, 106 cells together with the same number of irradiated syngeneic thymocytes were cultured in the presence of the virus-specific antigen per ml for 5 days. Thereafter, 5 × 104 irradiated syngeneic thymocytes were cultured together in Iscove's modified Dulbecco medium (IMDM) supplemented with 15% interleukin-2 (IL-2)-containing medium and 5% rat serum in 6-day cycles together with 5 × 104 irradiated syngeneic thymocytes in the presence of the virus-specific antigen. After the fifth restimulation in vivo, when sufficient numbers of cells and stable cultures had been established, the cells were restimulated in the presence of either purified p24 plus p40 virus-specific proteins or recombinant p24 or recombinant p40 alone. In all cases, the T-cell cultures were restimulated at least once with a new batch of IL-2.

Transfection of the BDV-specific T-cell line. Various numbers of BDV-specific CD4+ T cells (5 × 103 to 5 × 106) were injected intravenously into the tail veins of rats at different time points prior to BDV infection.

Proliferation assay. To determine the antigen specificity of the T-cell cultures, proliferation assays were performed. Therefore, 5 × 104 T cells were cultured in the presence of 5 × 104 irradiated syngeneic thymocytes with 30 μg of recombinant p24 or p40 (provided by W. I. Lipkin, Irvine, Calif.) per ml BDV-specific protein, and influenza virus nucleoprotein (provided by H. Weckerle, Munich, Germany) or virus-infected (BDV-F10) and noninfected (F10) histocompatible astrocytes (the astrocytic cell line cloned from a primary Lewis astrocyte culture is kindly provided by H. Weckerle, Munich, Germany) or virus-infected (BDV-Lou) and noninfected (Lou) histocompatible skin fibroblasts from Louvain rats were labeled with 0.2 μCi of 35S]methionine for 3 h at 37°C for 1 h and washed three times with medium. Target cells were incubated with various effector:target ratios in a final volume of 200 μl/well. In some experiments, target cells were pretreated with rat gamma interferon (INF-γ) for 72 h to induce expression of MHC class II antigen (20). Some tests were performed in the presence of MAb directed against MHC class I (OX-18) or MHC class II (OX-6) (both from Serotec, Cambridge, United Kingdom) determinants. After 9 h, 50 μl of sample was collected and counted in a gamma counter. The percentage of [35S]methionine release was calculated according to the following formula: 100 × ([test release − spontaneous release]/spontaneous release), where test release is in the presence of effector cells, spontaneous release is in the presence of medium alone, and maximal release is in the presence of 1 N HCl.

RT-PCR analysis. For reverse transcription-PCR (RT-PCR), total cellular RNA was isolated from brain homogenates of BDV-infected rats. BDV-infected rat brains were used to separate mRNA from the total cellular RNA according to a procedure described by the manufacturer. RNA was reverse transcribed with oligo(dT) primer and murine leukemia virus reverse transcriptase before resuspension to a final volume of 20 μl. Reverse-transcribed mRNA was amplified in a 100-μl reaction mixture containing 70 ng/gene oligonucleotide primer per μl, 10 mM (each) dATP, dCTP, dGTP, and dUTP (Pharmacia, Freiburg, Germany), 500 mM KCl, 250 mM Tris-HCl (pH 8.3), 100 mM MgCl2, and 5 U of Ampli-Taq DNA polymerase (Amersham) per μl. The reaction was performed in a Biometra thermocycler for 35 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. An additional 10 μl of the reaction mixture was loaded onto a 1% agarose minigel and visualized by ethidium bromide staining.

For the following primers, amplification of mRNAs of BDV, CDS, perforin, and cytokines, have been described as previously (21, 28). BDV p40 antisense, 5′-GGTTGAGCATCTCATACATTCTGCAGAG-3′; BDV p40 sense, 5′-CAGTAGACCCGACCCTGGTTGC-3′; CD8 antisense, 5′-CATGAAGTGAATCCTGGCTCCTCCG-3′; CD8 sense, 5′-CTCTTCGCAGCTCTGTCTC-3′; perifosine antisense, 5′-CCGGGATGTGAATGTTGC-3′; perifosine sense, 5′-AGCCCTGGCACACATCCG-3′; β-actin antisense, 5′-GACATTGCCGGTGACAGATGGAG-3′; and β-actin sense, 5′-ATGCGCTTGGCTGCACCCTG-3′. The sensitivity of our assay had a titer of 104 to 105 molecules of in vitro-synthesized BDV p40 RNA in 1 ml. Samples of uninfected rat brain were used as negative controls. As a positive RNA control, primers for β-actin were used.

Cytofluorometry. Unstained and stained T-cell lines were scanned on an Epics Elite laser flow cytometer (Coulter Electronics, Hialeah, Fla.). During acquisition, the T-cell population was gated to exclude debris and 105 cells were counted per sample. Cells were incubated with various fluorescein isothiocyanate-conjugated antibodies specific for the following mouse differentiation markers (Camon Immunology, Wiesbaden, Germany): W3/13 (T cells), OX-33 (B cells), W3/25 (CD4+ T cells), OX-8 (CD8+ T cells), and P 12520 (anti-CD49d; α4-integrin) and R73 (CD8+ T-cell receptor) (Dianova).

In vivo hybridization. Digoxigenin-labeled RNAs complementary to BDV mRNAs were prepared from the BDV clone pAF4 (kindly provided by W. I. Lipkin). Brains from experimental animals were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Five-micrometer sagittal sections were mounted on slides, and paraffin was removed with xylene. After treatment with proteinase K and 0.5 N HCl to facilitate penetration of the probe, hybridization was carried out overnight at 65°C with 20 ng of probe per slide by the standard protocol (Boehringer, Mannheim, Germany).

Histology and immunohistochemistry. Immediately after the rats were killed at different time points after infection, brain samples were obtained. Materials were either frozen in isopentane at −150°C or fixed in buffered paraformaldehyde. All tissue sections were stained with hematoxylin and eosin. Encephalitic infiltrates were scored with an arbitrary scale ranging from 0 to 3, based on the number of infiltrates per section and the number of cell layers in each infiltrate (1, up to 5 small infiltrates/section; 2, more than 5 small infiltrates/section or more than 3 infiltrates with multiple layers; 3, more than 10 small infiltrates or more than 5 infiltrates with multiple layers). Immunohistochemistry was carried out on cryostat sections for the presence of lymphocyte subsets and macrophages and microglia. The following MAb were used: OX-8 (anti-CD8+ T cells), OX-38 (anti-CD4+ T cells), and ED1 (macrophages) (Serotec) and anti-tumor necrosis factor alpha and anti-IFN-γ (Genzyme, Cambridge, Mass.).

RESULTS

Cultivation and characterization of BDV-specific CD4+ T-cell line K38.24. Lymphocytes from the political lymph nodes of Lewis rats immunized with a mixture of affinity-purified BDV-specific antigen containing the two major proteins p40 and p24 were harvested 11 days after local immunization. Lymphocytes were repeatedly restimulated in vitro in the presence of irradiated syngeneic thymocytes and BDV-specific antigen containing either both major virus-specific proteins or only p40 or p24. To mimic the situation in vivo, for this study we chose a T-cell line (K38.24) that was maintained in the presence of both virus-specific proteins. The specificity of this T-cell line was determined in proliferation assays at various time points of cultivation in the presence of virus-specific proteins. In all of the experiments described, T cells from the 7th through the 13th restimulation cycle, when stimulation indices (SI) for both virus-specific proteins remained high (SI of 41 to 55), were used. Treatment with proteinase K and 0.5 N HCl did not induce proliferation (SI of 1; data not shown). Furthermore, the K38.24 T-cell line was phenotypically characterized in fluorescence-activated cell sorter analyses, revealing the phenotype of a CD4+ T-cell line, namely,
of this T-cell line included the determination of the cytokine profile by RT-PCR analysis and revealed the presence of IL-2, IL-4, and IFN-γ but not IL-6 or IL-10 (data not shown). Most importantly, cytotoxicity assays on a persistently BDV-infected astrocytic target cell line (BDV-F10) uniformly revealed the absence of lytic activity, even after IFN-γ treatment of target cells to upregulate MHC class II antigen expression in BDV-infected cells (data not shown) (20). Characterization of the CD4⁺ T-cell line K38.24 in vivo revealed the induction of typical BD symptoms after adoptive transfer into BDV-infected immunosuppressed recipient rats (data not shown).

**T-cell treatment prior to infection results in absence of disease symptoms and elimination of virus.** Various protocols were followed in T-cell treatment experiments with T-cell line K38.24. In the first experiment, 5 × 10⁵ cells were used for transfusion at day 12 before intracranial (i.c.) challenge and 2 × 10⁶ cells were used for day 2 before i.c. challenge (Fig. 1 and Table 1). The treated rats showed a faster antibody kinetic, no disease symptoms, and an early onset of a slight inflammatory reaction during the 20-day observation period (Table 1). Health status is reflected by an increase in body weight after day 14, when infected control rats showed decreases in body weight (Fig. 1A), moderate to severe clinical symptoms, and a strong encephalitic reaction (Table 1). At both time points, the virus titers in the brains of T-cell-pretreated rats were considerably lower than those in untreated infected controls (Fig. 1B).

Next, K38.24 CD4⁺ T cells were transfused into naïve rats 13 days (2 × 10⁶ or 3 × 10⁶ cells) and again 3 or 2 days (10⁶ cells) before rats were infected i.c. with BDV, and the observation period was prolonged (Fig. 2 and Table 2). Whereas control animals showed the beginnings of clinical disease after day 14 and full-blown disease at later time points, T-cell-treated rats had clinical scores that were transiently very low or exhibited no clinical symptoms (Tables 2 and 3). Interestingly, in T-cell-treated rats, a low-level encephalitic reaction was again seen by day 9, whereas the controls did not show any evidence of inflammatory cell aggregation at this time point. The body weight curves reflect the rats’ general health status. BDV-infected control rats had steadily decreasing body weights, whereas rats that received T cells showed only a transient weight loss or even increased body weights (Fig. 2A and 3A). The rats were killed at various time points after infection and were tested for the presence of pathological alterations and virus in the brain.

The inflammatory reactions in the brains of rats that received T cells prior to infection were in general less pronounced and never received a score higher than 1 (see Materials and Methods). However, both T-cell-treated rats killed at day 9 after infection had a low-level encephalitic reaction with CD4⁺ and CD8⁺ T cells, whereas the infected control rats did not (Table 2). At later time points, infected controls without T-cell treatment exhibited significant brain inflammation and obvious clinical signs of BD (Tables 2 and 3). Most interest-

![Graph A](image1.png)

**Graph A.** Transfusion of CD4⁺ T-cell line at days 12 (5 × 10⁵) and 2 (2 × 10⁶) before BDV infection. (A) Body weight curves for T-cell-treated rats (■) and untreated control rats (▲). (B) Virus titers (mean of values listed in Table 1) for T-cell-treated rats (❖) and untreated control rats (▲). Virus titers are given as log₁₀.

![Graph B](image2.png)

**Graph B.** Virus titer in brain.

**TABLE 1.** Data for transfusion of CD4⁺ T-cell line at days 12 (5 × 10⁵) and 2 (2 × 10⁶) before BDV infection

<table>
<thead>
<tr>
<th>T-cell transfer</th>
<th>Day p.i.</th>
<th>Symptoms</th>
<th>Encephalitis</th>
<th>Antibody titer (log₂)</th>
<th>Virus titer (log₁₀)</th>
<th>Presence of mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>8</td>
<td>0, 0, 0</td>
<td>0, 0</td>
<td>&lt;1, &lt;1, &lt;1</td>
<td>2.5, 3.0, 3.8</td>
<td>+, +, +</td>
</tr>
<tr>
<td>+</td>
<td>8</td>
<td>0, 0</td>
<td>0.5, 0.5</td>
<td>2.5</td>
<td>10, 1.7</td>
<td>+, +</td>
</tr>
<tr>
<td>−</td>
<td>20</td>
<td>0.5, 1.5, 1.0</td>
<td>1.5, 1.5, 2.0</td>
<td>6, 7, ≥8</td>
<td>5.9, 5.9, 5.9</td>
<td>+, +, +</td>
</tr>
<tr>
<td>+</td>
<td>20</td>
<td>0, 0</td>
<td>0.25, 0.5</td>
<td>≥8, ≥8</td>
<td>1.7, 2.5</td>
<td>+, +</td>
</tr>
</tbody>
</table>

* Data are for individual rats. Encephalitis and clinical scores were on a scale ranging from 0 to 3.
infected rats (Fig. 2A and 3A; Tables 2 and 3). Also, depending on the time period after infection, T-cell-treated rats lacking virus in the brain exhibited virus-specific antigen below detectable levels or at drastically reduced levels, as demonstrated by staining of rare single cells, compared to untreated infected controls showing widespread dissemination of virus-specific antigen (Fig. 4A through D). Corresponding with these findings, in general, no virus-specific mRNA was detected by RT-PCR in the brains after day 23 after infection (Tables 1 to 3), and the same was true for in situ hybridization experiments (Fig. 4E and F). In addition, the morphological structure in the brains of T-cell-treated rats was not altered visibly, whereas infected control rats had severe degenerative defects in the cortical areas (Fig. 4G and H). Most strikingly, immunohistochemical examination also revealed that in all T-cell recipients, including those in which virus had been eliminated from the cortex, virus-specific antigen and an inflammatory reaction were restricted apparently exclusively to the hippocampal area, but no overt disease was seen (data not shown). In total, 23 rats were tested for the presence of virus after T-cell treatment, and they generally had reduced virus titers (n = 14) or no virus (n = 6) in the brain (data for 19 rats are shown in Fig. 1 to 3 and Tables 1 to 3). Treatment with the described CD4+ T-cell line resulted in protection from severe encephalitis and disease and in clearance of virus from the brain.

Elimination of virus from the brain correlates with the early presence of CD8+ T cells and perforin. In earlier studies we showed that virus-specific CD8+ T cells are present in the brains of BDV-infected and otherwise untreated rats (4, 20, 28). Therefore, we investigated the appearance of CD8+ T cells in the brains of T-cell-treated rats versus those of control rats. Though only CD4+ T cells had been used for treatment, numerous CD8+ cells as well as CD4+ T cells were found in all recipient rats (Fig. 5A and B and Tables 1 to 3). At very early time points after infection (day 6, Table 3), no CD8 mRNA was detectable in any rat tested. Slightly later, CD8+ T cells were detected in treated but not in control rats by RT-PCR specific for the mRNA of CD8 (day 8, Table 1; day 9, Table 2; day 10, Fig. 5B). Furthermore, at late time points after challenge, in the absence of infectious virus and virus-specific mRNA, very few (day 20, Table 1 and Fig. 5C) or no (day 32, Table 3) CD8+ T cells were found in the brain.

Since perforin has been identified as the major effector molecule in cytolysis by CD8+ T cells, and since we have demonstrated the presence of mRNA for perforin in BDV-infected rats (28), we tested the brains of T-cell-treated and untreated infected rats for the presence of perforin mRNA by RT-PCR. First, in T-cell recipients there was a clear correlation between the early presence of CD8+ T cells and perforin mRNA in the brain (Tables 1 and 2). When no CD8 mRNA was detectable, no perforin mRNA was found either (Table 3). In some cases, discrepancies between the kinetics of the presence of CD8+ T

---

**TABLE 2. Data for transfusion of CD4+ T-cell line at days 13 (2 × 10⁶) and 3 (1 × 10⁶) before BDV infection**

<table>
<thead>
<tr>
<th>T-cell transfer</th>
<th>Day p.i.</th>
<th>Symptoms</th>
<th>Encephalitis</th>
<th>Antibody titer (log₂)</th>
<th>Virus titer (log₁₀)</th>
<th>Presence of mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>&lt;1, &lt;1</td>
<td>2.5, 3.0</td>
<td>+, +</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0</td>
<td>0.5, 1.0</td>
<td>1, 4</td>
<td>2.5, 3.0</td>
<td>+, +</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0, 0.5</td>
<td>0.5, 1.0</td>
<td>6, ≥8</td>
<td>3.0, 5.3</td>
<td>+, +</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.5, 0.5</td>
<td>0.5, 1.0</td>
<td>≥8, ≥8</td>
<td>1.0, 3.0</td>
<td>+, +</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>1.5, 1.5</td>
<td>1.5, 2.0</td>
<td>≥8, ≥8</td>
<td>5.3, 5.3</td>
<td>+, +</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>0, 0</td>
<td>0</td>
<td>≥8, ≥8</td>
<td>&lt;1.0, &lt;1.0</td>
<td>−, −</td>
</tr>
</tbody>
</table>

* Data are for individual rats. Encephalitis and clinical scores were on a scale ranging from 0 to 3.
cells and perforin in vaccinated and control rats appear to be of great importance (e.g., day 9, Table 2). In this case, RT-PCR analyses for CD8 and perforin mRNA produced positive results for both rats which received the CD4+ T-cell line, while results were negative for infected and untreated control rats. The results for day 8 after challenge were essentially the same (Table 1).

**Presence of CD8+ T cells and perforin correlates with cytotoxicity in T-cell-treated rats.** After having established the early presence of CD8+ T cells and the major effector molecule of cytotoxicity in CD4+ T-cell-treated rats, we questioned whether cytotoxic activity could be found in lymphocytes isolated from the brains of treated and untreated infected controls at early time points after infection. Therefore, lymphocytes isolated from the brains of Lewis rats after T-cell treatment and from infected Lewis controls on day 9 postinfection (p.i.) were tested by conventional cytotoxicity assays (Fig. 6). Cytotoxic activity was detectable in brain lymphocyte preparations isolated from infected and untreated control rats. The BDV-infected syngeneic astrocytic target cell line (BDV-F10) was moderately lysed after an 8-h incubation period by day 9 lymphocytes, but allogeneic BDV-infected cells (BDV-Lou skin cells) were not killed (Fig. 6, right panel). Pretreatment of target cells with IFN-γ to enhance the expression of MHC class II antigen of target cells did not result in increased lysis, even in a 16-h assay (data not shown). In contrast, day 9 brain lymphocyte preparations from CD4+ T-cell-treated rats elicited significantly higher lysis of BDV-infected syngeneic target cells (Fig. 6, left panel). Here again, despite the higher cytolytic capacity of lymphocytes from T-cell-treated rats, allogeneic infected target cells were not lysed, and treatment of syngeneic targets with IFN-γ did not change the results, i.e., it did not cause MHC class II-restricted lysis. Essentially the same results were obtained after the coincubation period was increased to 16 h to enable lysis from MHC class II-restricted killer cells due to the generally observed delayed kinetics for CD4+ T-cell-mediated killing in rats (data not shown). Again, lysis of target cells was comparable whether or not targets were treated with IFN-γ. These data confirm earlier results (20, 28).

To exclude a possible role of locally synthesized IFN-γ in virus elimination from the brain, we looked for the presence of this cytokine by immunohistochemistry with sections processed in parallel (Fig. 7). However, staining in T-cell-treated rats was even less intense than that in untreated infected rats.

**No evidence for importance of neutralizing antibodies after CD4+ T-cell vaccination.** In all T-cell-treated rats, the kinetics of virus-specific antibody synthesis were enhanced Tables 1 to 3). In most cases, titers as determined by ELISA were two- to fourfold higher than those for untreated infected controls. Since antiviral antibodies can have neutralizing activity which might interfere with virus replication or neutralize extracellular...
FIG. 4. Clearance of BDV from the brains of CD4+ T-cell-treated rats; reduced expression of BDV-specific p40 in T-cell-treated (A and C) versus untreated, infected control (B and D) rats at day 10 (A and B) and day 20 (C and D); in situ hybridization in the cortical area of T-cell-treated (E) and untreated, infected control (F) rats with a probe for p40 mRNA at day 32 p.i.; absence of BDV RNA in T-cell-treated rats. Note the uninfected, unstained pyramidal neurons in T-cell-treated rats (E) (arrowheads). In contrast, most neurons and many astrocytes are infected with BDV in untreated control rats (F). Almost-intact brain morphology of a T-cell-treated rat (G) at day 32 p.i. in the neocortex and inflamed brain with morphological alterations in an infected control rat (H). (A through D) Immunohistology with the anti-p40 specific MAAb 3H17C1; (E and F) in situ hybridization; (G and H) hematoxylin and eosin staining. Magnifications: A, B, E, F, and G, ×50; C, ×100; D, ×120; H, ×30.
lar virus, sera from rats transfused with the CD4$^+$ T-cell line and rats infected without T-cell treatment were tested in neutralization assays. Titers of neutralizing activity in all sera and rats infected without T-cell treatment remained below the detection level (NT$50 < 1:32$) independently of the antibody titers found in binding assays (ELISA). Therefore, we conclude that none of the infected control rats and none of the T-cell-treated and infected rats synthesized detectable neutralizing antibodies during the 32-day observation period. This finding is in good agreement with the earlier observation that neutralizing antibodies are detectable only after 10 to 15 weeks p.i. (12).

**DISCUSSION**

In this study we show that transfusion of virus-specific CD4$^+$ T cells results in the termination of a viral infection initiated after transfusion. These results support and extend earlier experimental data obtained in the same virus system by Richt et al. (23). Moreover, they provide a mechanistic basis for the understanding of the termination of a potentially persistent viral infection and protection from virus-induced immunopathology. We argue that the observed phenomena are not due to an effector mechanism mediated by transfused virus-specific CD4$^+$ T cells but, rather, present strong evidence for a virus-specific CD8$^+$ cytotoxic T-cell response induced by transfusion with a CD4$^+$ T helper cell.

During recent years, BDV infection of rats has been established as an important model of an immunopathological disease in the brain resulting in severe neurological symptoms such as abnormal behavior, disturbances of motility and, finally, signs of debility and dementia (5, 17, 29). These changes have been correlated with the initial invasion of the brain by mononuclear cells that results in severe inflammatory reactions throughout this organ as well as degenerative alterations of various cell types, including neurons (4, 20, 28, 30). Ultimately, a significant proportion of brain tissue, especially the cortex, is destroyed; this is represented by cortical brain atrophy (4, 17, 28). Since the basis of this disease is a persistent infection of the central nervous system, one could argue that the elimination of the virus and therefore the absence of viral replication and the lack of virus-specific antigens would also prevent immunopathology and disease. However, despite a vigorous T-cell-mediated immune response defined by the cytolytic activity of classical MHC class I-restricted T cells, the virus persists in the central nervous systems of infected individuals (7, 18, 20). In addition, the mere presence of virus-specific antibodies, even those with neutralizing activity, does not prevent infection or limit or eliminate the virus from the host (12). Therefore, the finding that the transfusion of virus-specific CD4$^+$ T cells prior to infection eliminates the virus and prevents disease appears to be rather important (23). However, the mechanism of this phenomenon remained unanswered. The following differences between the experiments reported by Richt et al. (23) and those described here may be important: first, the use of a T-cell line that induced cytolysis of MHC class II-bearing target cells and did not allow Richt et al. to decide whether the transfused cytolytic CD4$^+$ T cells or other mechanisms were responsible for the observed virus elimination; second, these authors did not employ methods to demonstrate or phenotype infiltrating cells such as CD4$^+$ or CD8$^+$ T cells; and third, these researchers did not include functional assays. In contrast to Richt et al., we have never found any argument for the operativeness of MHC class II-restricted cytolysis in this disease (20, 21, 28). Nevertheless, after transfusion with virus-specific CD4$^+$ T-cell lines that lack cytotoxic activity in vitro, we were able to induce BD (21; this study). We could show that these BD-specific T cells, obviously acting as helpers, were sufficient to cause disease via the recruitment of CD8$^+$ T cells to the brain, whereas recipient rats depleted of CD8$^+$ T cells or all T cells by T-cell-specific antibodies did not show neurological symptoms or destructive encephalitis (21). Therefore, in the study reported here, we decided to use the CD4$^+$ T-cell line K38.24, which does not induce cytolytic activity in vitro and therefore could not be directly responsible for the lysis of infected cells in vivo. However, the effect of CD4$^+$ T-cell transfusion prior to i.c. virus challenge on virus titers and clinical disease was quite impressive. If given CD4$^+$ T cells before virus challenge, rats were capable of limiting and even abrogating virus replication, resulting in reduced virus titers and even in an absence of detectable virus at later time points after challenge. In contrast, if given after infection, the same cell line was capable of inducing disease (data not shown). The

**FIG. 5.** Presence and localization of T-cell populations in CD4$^+$ T-cell-treated rats: perivascular distribution of CD4$^+$ T cells (A, day 10) and dissemination of CD8$^+$ T cells (B and C) in the brains of T-cell-treated rats. Note the difference in the numbers of CD8$^+$ T cells at day 10 (B) and day 20 (C). In infected control rats, very few cells are found at day 10 (data not shown). Magnifications: A, ×50; B, ×10; C, ×60.
lack of virus in T-cell-treated rats was demonstrated by the generally complete absence of infectious virus, virus-specific antigen, and virus-specific RNA as shown by RT-PCR and in situ hybridization. In all experiments, transient virus replication was seen in the brains of the challenged rats. At early time points after infection, virus titers in rats treated with T cells were equal to or lower than those from controls. These results are in agreement with the proposed role of cytotoxic T cells in BD; earlier, we provided several lines of evidence that CD8$^+$ T cells are involved in the immunopathogenesis and degenerative encephalopathy that occur after infection with BDV (5, 29). First, MHC class I-restricted lysis can be detected in lymphocytes isolated from the brains of diseased rats (20, 28). Second, the appearance of CD8$^+$ T cells in the brain coincides with the onset of disease and the destruction of brain cells (4, 28, 30). Third, the absence of CD8$^+$ T cells results in the prevention of disease (21, 30, 31). Fourth, the adoptive transfer of brain lymphocytes with high cytolytic capacity results in the early onset of severe degenerative alterations in the brain, as represented by spongiform degeneration; and fifth, cytodestruction is linked with the presence of perforin mRNA (perforin is a major pathway of CD8-mediated cellular destruction [28]).

In the experiments described in the present study, we found a direct correlation between the enhanced kinetics of CD8$^+$ T cells, MHC-restricted cytotoxicity, the presence of perforin mRNA, and the loss of virus from cortical brain areas compared to those in control rats exhibiting immunopathological reactions and disease. Interestingly, the time points when CD8$^+$ T cells and perforin mRNA could be detected in the brains of T cell-treated rats and untreated rats differed. In addition, rats receiving virus-specific CD4$^+$ T cells prior to infection had CD8$^+$ T cells predominantly in parenchymal locations, whereas CD4$^+$ T cells appeared to be stringently restricted to perivascular locations, as demonstrated by immunohistochemistry. This finding again supports our concept and is in agreement with the distribution patterns of T cells at early time points after infection, when the local activity of CD8$^+$ T cells commences. Furthermore, considerably more CD8$^+$ T cells were found in cortical areas of T-cell recipient rats at 10 days p.i. than at day 20 or 32, when the virus load was drastically reduced or even eliminated. In this respect, it is worth noting that the time courses of IFN-γ and perforin expression correlated.

FIG. 6. Nine-hour cytotoxicity assay with lymphocytes isolated from the brain of a T-cell-treated rat (left panel) versus an untreated infected control rat (right panel) at day 9 p.i. Spontaneous release of syngeneic BDV-F10 (24%) and allogeneic BDV-Lou (Louvain, 19%). E:T, effector cell to target cell.

FIG. 7. Immunohistological detection of IFN-γ in T-cell-treated (A, ×50) and untreated control (B, ×30) rats. Note the lower staining activity for IFN-γ (arrowheads) in T-cell-treated rats in panel A. Magnifications: A, ×50; B, ×30.
CD4 T-CELL TREATMENT INDUCES PROTECTIVE CTL RESPONSE

Volume 72, 1998

4395

CD4 T-cell treatment induces protective CTL response

ACKNOWLEDGMENTS

This work was supported by grants from the Deutsche Forschungs-
gemeinschaft (Sti 71/2-1 to L.S., Sti 71/2-2 to L.S. and O.P., and Bi 323/2-2 to T.B.) and an EU grant (CHRX-CT94-0670 to L.S.). We thank Martin Sobbe for help and valuable discussions and Silke Gommel for outstanding technical assistance.

REFERENCES


