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Induction of Degenerative Brain Lesions after Adoptive Transfer of Brain Lymphocytes from Borna Disease Virus-Infected Rats: Presence of CD8$^+$ T Cells and Perforin mRNA

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Lymphocytes were isolated from the brains of Borna disease virus-infected donor Lewis rats at various time points after infection. Cell populations were characterized by cytofluorometry, with special emphasis on CD4$^+$ and CD8$^+$ cells. Testing of isolated lymphocytes revealed major histocompatibility complex class I-restricted cytotoxic activity. Reverse transcription-PCR analyses of brain homogenates of infected donors revealed the presence of CD8 mRNA after day 11 of infection and of perforin mRNA between days 13 and 25 after infection. Adoptive transfers of lymphocytes isolated from the brain at days 13 and 21 resulted in severe neurological symptoms, resembling experimental Borna disease. The onset of disease was dependent on the cell numbers transferred and was clearly related to the appearance of T cells in the brain. CD8$^+$ T cells were found in the parenchyma, whereas CD4$^+$ T cells were found predominantly in perivascular locations. A disseminated lymphocytic infiltration in the parenchyma was accompanied by severe morphological alterations, including significant necrosis of neurons. Furthermore, a prominent spongiform-like degeneration was observed; this increased over time and finally resulted in severe cortical brain atrophy. Lymphocytes obtained during the beginning chronic phase of experimental Borna disease in rats had no significant cytolytic capacity in vitro and were also not able to induce neurological symptoms typical of Borna disease after adoptive transfer. The data presented here show for the first time that lymphocytes isolated from the site of the inflammatory lesions, namely, the brains of diseased rats, induce the immunopathological reaction and cause Borna disease. After transfer, the pathological alterations induced in the recipients exactly reflect those observed during experimentally induced Borna disease in rats, including necrosis of neurons and glial cells and gross degeneration resulting in cortical brain atrophy. Evidence that the immunopathology of Borna disease is closely related to the presence of CD8$^+$ T cells in the brain parenchyma is provided.

Borna disease (BD), which is caused by the negative-single-stranded RNA BD virus (BDV) (5, 6), is a naturally occuring neurological disease in horses, sheep, cattle, and possibly other species of animals (19, 27, 34). Furthermore, evidence that BDV might be a human pathogen involved in psychiatric disorders has been presented (4, 28, 39). Experimental BD represents an interesting model of T-cell-mediated immunopathology in the brain (reviewed in references 3 and 32). Both CD4$^+$ and CD8$^+$ T cells have been shown to participate in this immunopathological reaction, which results in severe neurological symptoms in naturally or experimentally infected hosts (1, 21, 26, 35, 36). Whereas CD4$^+$ T cells appear to act predominantly as T helper cells, there is ample evidence that CD8$^+$ T cells act as effector cells, because (i) the elimination or functional blocking of CD8$^+$ T cells results in the absence of neurological disease symptoms (33, 35) and prevents morphological alterations in the brain (2, 22), and (ii) the destruction of brain cells was related to the presence of major histocompatibility complex (MHC) class I-restricted T cells which exerted cytotoxic activity upon in vitro testing (22). Other mechanisms involved in brain pathology after BDV infection have been proposed, including the actions of nitric oxide and proinflammatory cytokines (10, 16, 30, 40). The activity of CD4$^+$ T cells as effector cells in this virus-induced degenerative encephalopathy appears to be rather limited, although cytotoxic activity has been found in a particular BDV-specific CD4$^+$-T-cell line (26). Other CD4$^+$-T-cell lines do not exhibit cytolysis of virus-infected target cells and alone do not cause disease or degenerative alteration after adoptive transfer into BDV-infected immunosuppressed recipients (23). However, adoptive transfer of noncytolytic CD4$^+$ T cells results in disease if CD8$^+$ T cells enter the brain (23).

Since T-cell lines cultured for long periods of time might not totally reflect the in vivo situation, we have performed experiments in which lymphocytes were directly isolated from the brain and were characterized phenotypically by cytofluorometry and functionally by cytotoxicity assays before being used in adoptive transfer experiments. The results clearly show the importance of CD8$^+$ T cells or CD8$^+$-T-cell-mediated mechanisms for pathogenesis and brain cell destruction in this model of a neurodegenerative disease.

MATERIALS AND METHODS

Virus and experimental animals. The Giessen strain He/80 of BDV was used for this study. Female Lewis rats obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany) were infected at the age of 5 weeks by injection into the left hemisphere with 5×10^3 50% tissue culture infective doses ($TCID_{50}$) of BDV.

Clinical evaluation. All experimental animals were examined daily and weighed, and disease symptoms were scored by two independent observers on an

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arbitrary scale from 0 to 3 based on the general state of health and the appearance of neurological symptoms (1, slight incoordination and fearfulness; 2, distinct ataxia or slight paresis; 3, paresis or paralysis). The weight at the day of infection was considered 100%, and the percent weight change was calculated.

Infectivity assay and antigen detection. Assays were done essentially as described before (33). Briefly, the infectivity of virus from brain homogenates was determined on rabbit embryo brain indicator cells by indirect immunofluorescence with rat hyperimmune sera or a BDV-specific monoclonal antibody (MAb) (37).

Antibody titration. All sera were tested in twofold dilutions in a solid-phase enzyme-linked immunosorbent assay and in Western blot analysis with a purified antigen from BDV-infected rats (36).

RT-PCR analysis. For reverse transcription-PCR (RT-PCR), total cellular RNA was isolated from brain homogenates of BDV-infected rats. Magnetic beads (Dynabeads; Dianova, Hamburg, Germany) were used to separate the mRNA from the total cellular RNA according to a procedure supplied by the manufacturer. RNA was reverse transcribed with an oligo(dT) primer and murine leukemia virus reverse transcriptase before being resuspended to a final volume of 20 μl. The reverse-transcribed mRNA was amplified in a 100-μl reaction volume containing 70 ng of each oligonucleotide primer per μl; 10 mM (each) dATP, dTTP, dGTP, and dCTP (Pharmacia, Freiburg, Germany); 500 mM KCl; 250 mM Tris-HCl, pH 8.3; 100 mM $MgCl_2$; and 5 U of Ampli-Taq DNA polymerase (Amersham) per μl. The reaction was performed in a Biometra thermocycler for 35 cycles of 95°C for 1 min, 65°C for 2 min, and 72°C for 3 min, after which 10 μl was loaded onto a 1% agarose minigel and visualized by ethidium bromide staining.

Alternatively, nested RT-PCR was done to increase the sensitivity of detection of the amplification products. For this procedure, two consecutive PCRs, each involving 35 cycles of amplification, were utilized. For the first PCR an external pair of primers was used, while for the second PCR two nested primers which were internal to the first primer pair were used. The larger fragment produced by the first reaction was used as template for the second PCR. Furthermore, hot-start PCR was also done to increase the sensitivity of the amplification. In this procedure the polymerase was added after denaturation and preheating of the sample to 80°C to avoid unspecific primer hybridization.

In addition to the primers used in an earlier study (23), the following primer sequences were used: CD8 antisense, 5′-CATGAAGTGAATCCGGGCTCTC-CTCCGC-3′; CD8 sense, 5′-CTCCTTCAGACTCCTTCATCCCTGCTGGTT-3′; perforin antisense, 5′-CCGGGGATTGTTATTGTTCC-3′; and perforin sense, 5′-AGCCCCTGCACACATTACTG-3′.

Isolation of effector cells. Lymphocytes from the brain were isolated by a method previously described by Irani and Griffin (11) and modified for the BDV infection of rats (22). The animals were anesthetized with ketamine hydrochloride and perfused with balanced salt solution. The brain was homogenized carefully through a stainless steel mesh and collected in balanced salt solution containing collagenase D (0.05%), trypsin inhibitor (TLCK [*N*α-*P*-tosyl-L-lysine chloromethyl ketone]) (0.1 μg/ml), DNAse I (10 μg/ml), and HEPES (10 mM). The cell suspension was stirred at room temperature for 1 h and allowed to settle for 30 min. The supernatant was pelleted at 200 × *g* for 5 min in 10 ml of Ca- and Mg-free phosphate-buffered saline. Five milliliters of the suspension was layered on top of 10 ml of a modified RPMI-Ficoll gradient and centrifuged at 500 × *g* for 30 min. The pellet containing the lymphocytes was resuspended in Iscove's modified Dulbecco medium with 2% fetal calf serum, and the cells were counted for further use in transfer or cytotoxicity assays. Lymphocytes from spleens and cervical lymph nodes were obtained after homogenizing lymphoid tissue carefully through a steel mesh, and single-cell suspension were used without further treatment.

In vitro cell-mediated cytotoxicity. Aliquots of 10^7 virus-infected (BDV-F10) and noninfected (F10) histocompatible astrocytes (the astrocytic cell line cloned from a primary Lewis astrocyte culture was kindly provided by H. Wekerle, Munich, Germany) were labeled with 0.2 mCi of ^{51}Cr at 37°C for 1 h and washed three times with medium. Target cells were coincubated with effector cells from BDV-infected rats at various effector cell/target cell (E/T) ratios in a final volume of 200 μl/well. In some experiments, target cells were pretreated with rat gamma interferon (IFN-γ) for 72 h to induce expression of MHC class II antigen (22, 25). Some tests were performed in the presence of a MAb directed against MHC class I or class II determinants. After 9 h, 50 μl of sample was collected and radioactivity was counted in a gamma counter. The percent ^{51}Cr release was calculated according to the formula 100 × (test release − spontaneous release)/(maximal release − spontaneous release), where test release is release in the presence of effector cells, spontaneous release is release in the presence of medium alone, and maximal release is release in the presence of 1 N HCl. Spontaneous release never exceeded 25%.

Immunosuppression with cyclophosphamide. One day after intracerebral BDV infection, the animals received 160 mg of cyclosphosphamide per kg given intraperitoneally (21). After immunosuppression or antibody treatment, infected animals developed no signs of BD.

Adoptive transfer of lymphocytes. For adoptive transfer studies, lymphocytes directly isolated from brains, cervical lymph nodes, or spleens were injected intravenously (i.v.) into cyclophosphamide-treated recipients without further in vitro cultivation.

TABLE 1. Fluorocytometric analysis of lymphocytes isolated from the brains (BrLy), cervical lymphnodes (CLn), and spleens (SPL) of BDV-infected rats at various time points after infection

Lymphocytes	CD4/CD8 ratio[a] on day p.i.:		
	13	21	35
BrLy	1.0 ± 0.3:1	1.4 ± 0.4:1	1.7 ± 0.1:1
CLn	3.3 ± 0.5:1	3.3 ± 0.5:1	ND[b]
SPL	3.3 ± 0.5:1	3.3 ± 0.5:1	ND

[a] Values are means and standard deviations from four independent experiments.

[b] ND, not determined.

Histology and immunohistochemistry. Immediately after the animals were killed at different time points after infection, samples of rat brains 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 on 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/microglia. The following MAbs were used: OX-8 (anti-$CD8^+$ T cells), OX-38 (anti-$CD4^+$ T cells), and ED1 (macrophages) (Serotec, Cambridge, United Kingdom) and anti-tumor necrosis factor alpha and anti-IFN-γ (Genzyme).

Cytofluorometry. Unstained and stained lymphocytes were scanned on an Epics Elite laser flow cytometer (Coulter Electronics, Hialeah, Fla.). During the acquisition, the T-cell population was gated to exclude debris, and 10^4 cells were counted per sample. Cells were incubated with the following fluorescein isothiocyanate-conjugated MAbs specific for leukocyte differentiation markers: W3/13 (T cells), OX-33 (B cells), W3/25 ($CD4^+$ T cells), and OX-8 ($CD8^+$ T cells) (Camon, Wiesbaden, Germany) and P 12520 (VLA-4) and R73 (T-cell receptor α/β) (Dianova).

RESULTS

Characterization of brain lymphocytes. Cells were isolated from the brains of virus-infected rats at various time points after infection. Approximately six times more cells were recovered from the brains of rats on day 21 (average of 4×10^6/rat) than from rats on day 13 (average of 7×10^5), and twice as many were recovered as compared to rats on day 35 (average of 2×10^6). Phenotypic characterization of pools of these brain cells in cytofluorometric analyses revealed a ratio of CD4 to CD8 cells of 1:1 on day 13 and of 1.4:1 on day 21 (Table 1). The CD4/CD8 cell ratio in the brain reflects the high proportion of $CD8^+$ T cells in this location, whereas the usual ratios (ca. 3:1) were found in cervical lymph nodes and spleen cells.

Cytotoxicity assays reproducibly revealed cytolytic activity from lymphocytes isolated at days 13 and 21 postinfection (p.i.) (Fig. 1). The E/T ratios had been corrected for the number of $CD8^+$ cells in the cell suspensions according to the data obtained by cytofluorometry. Treatment of target cells with IFN-γ to increase the expression of MHC class II antigen did not result in an increase of cytolysis of target cells (Fig. 1A). Even higher E/T ratios for $CD4^+$ T cells and prolongation of assay times to 18 h did not change the results. To further determine the type of cytotoxic T lymphocyte responsible for the cytolytic effect observed, target cells were treated with either antibodies against MHC class I (OX-18) (Fig. 1B) or antibodies against MHC class II (OX-6) (Fig. 1C and D). Whereas the presence of anti-MHC class I antibodies greatly reduced lysis of target cells, the addition of anti-MHC class II antibodies had no effect. The latter experiments were exclusively performed with cells from day 21, since they were considerably more numerous and fewer rats were necessary to obtain sufficient numbers of cells from the brain. Cytotoxic

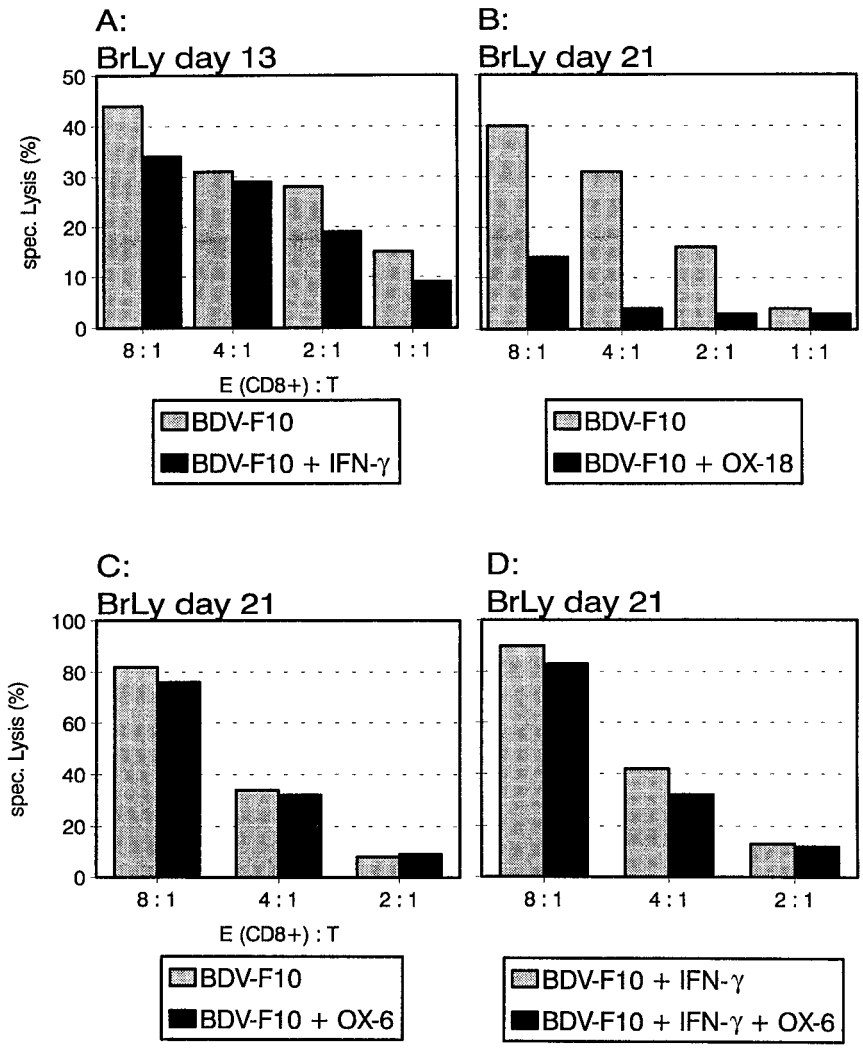


FIG. 1. Specific (spec.) lysis by lymphocytes isolated from the brains of BDV-infected rats tested on an astrocytic cell line. (A) Day 13 brain lymphocytes (BrLy) on MHC class I-bearing (BDV-F10) and MHC class II-bearing (BDV-F10 + IFN-γ) cells; (B) day 21 lymphocytes on MHC class I-bearing cells (BDV-F10) and after blocking of recognition by anti-MHC class I antibodies (OX-18); (C) day 21 lymphocytes on BDV-F10 targets and lack of reduction of lysis after treatment of target cells with anti-MHC class II antibodies (OX-6); (D) day 21 lymphocytes on MHC class II-expressing, IFN-γ-treated astrocytic target cells and lack of reduction after treatment with anti-MHC class II antibodies.

activity from lymphocyte preparations obtained at day 35 p.i. was not significant in most rats tested (see below).

Detection of CD8 and perforin mRNAs in brains of BDV-infected rats. In earlier reports (2, 22) we have demonstrated the presence of $CD8^+$ T cells in the brain parenchyma of virus-infected rats in parallel to the expression of MHC class I antigen in the brain by employing immunohistochemical methods. Here we attempted to detect mRNA for perforin, which is regarded to be the most important effector molecule in CD8-mediated lysis of target cells. As shown in Fig. 2, upregulation of perforin mRNA is detectable in virus-infected and otherwise untreated rats after day 13 p.i.. CD8 mRNA was already found at day 11 and lasted beyond day 35, whereas no message was seen for perforin at this late time point. These results agree with those of one experiment in which the cytotoxic activity of brain lymphocytes isolated from individual rats was tested, revealing that most rats did not have cytotoxic-T-cell activity at day 35 p.i. whereas a very few still had such activity (data not shown).

Adoptive transfer of brain lymphocytes causes BD. So far, for passive transfer experiments exclusively spleen lymphocytes or cultured T-cell lines had been employed. Therefore, it was of great interest to determine the biological function of lymphocytes directly isolated from the site of the inflammatory reaction upon adoptive transfer into BDV-infected immunosuppressed recipients, after having established a functional effect of T cells in BDV-infected donor rats by detecting MHC class I-restricted cytotoxicity in vitro and the presence of perforin mRNA in vivo.

In the first set of experiments, a large number of cells which had been obtained from rats at day 21 p.i. were transferred (Fig. 3). Either 4×10^6 $CD8^+$ T cells (group 1) or 1.5×10^6 $CD8^+$ T cells (group 2) were transferred i.v. into BDV-infected immunosuppressed rats. The recipients showed clinical symptoms such as disturbances of balance, ataxia, and the beginning of a steep decline of body weight on day 7 posttransfer (p.t.) at the latest and paresis and paralysis thereafter, whereas infected immunosuppressed control rats showed no

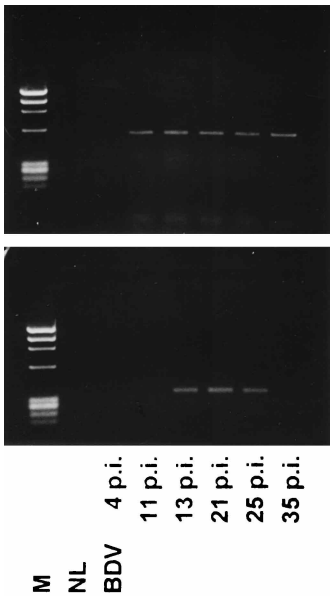


FIG. 2. Results of RT-PCR from brain homogenates of BDV-infected rats at various time points after infection. (Upper panel) Detection of CD8 mRNA; (lower panel) detection of perforin mRNA. M, marker; NL, normal uninfected brain.

symptoms and no loss of body weight. Further experiments were performed to establish whether lymphocytes from day 13 or lower numbers of lymphocytes from day 21 were capable of causing an immunopathological disease in immunosuppressed recipients upon adoptive transfer. As shown in Fig. 4, a total of 1×10^6 cells from either day 21 or day 13 (equaling 3.3×10^5 and 2×10^5 $CD8^+$ cells, respectively) were transferred, and this caused encephalitis and disease with some delay as compared to recipients which had received higher cell numbers. The body weight curves exactly reflect the onset and severity of disease in the recipients, whereas immunosuppressed controls

A

group	BrLy	total number	CD4+	CD8+	no. of recipients
1	day 21	$2x10^7$	$7x10^6$	$4x10^6$	3
2	day 21	$7x10^6$	$2,5x10^6$	$1,5x10^6$	5
3	-	-	-	-	3

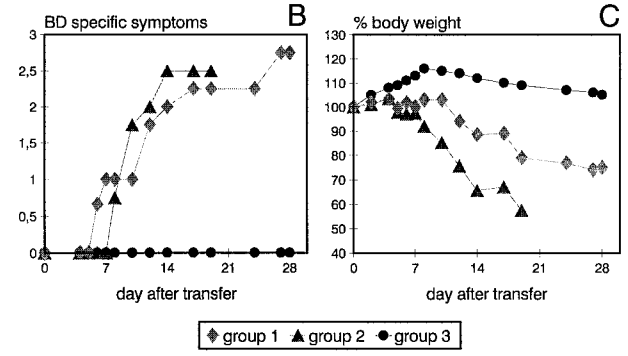


FIG. 3. (A) Adoptive transfer of brain lymphocytes (BrLy) isolated at day 21 p.i. into cyclophosphamide-treated, BDV-infected recipients. (B) Severity of clinical symptoms after transfer. (C) Decrease of body weight after transfer.

A

group	BrLy	total number	CD4+	CD8+	no. of recipients
1	day 13	$1x10^6$	$2,4x10^5$	$3,3x10^5$	3
2	day 21	$1x10^6$	$2,5x10^5$	$2x10^5$	3
3	-	-	-	-	3

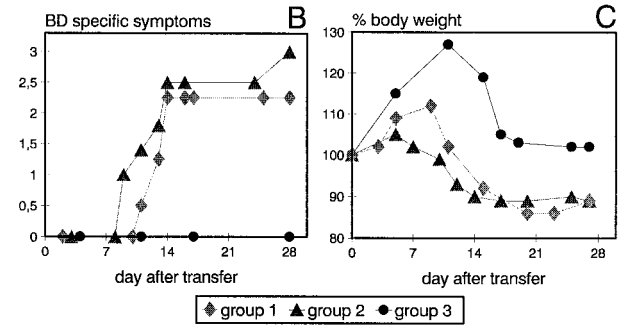
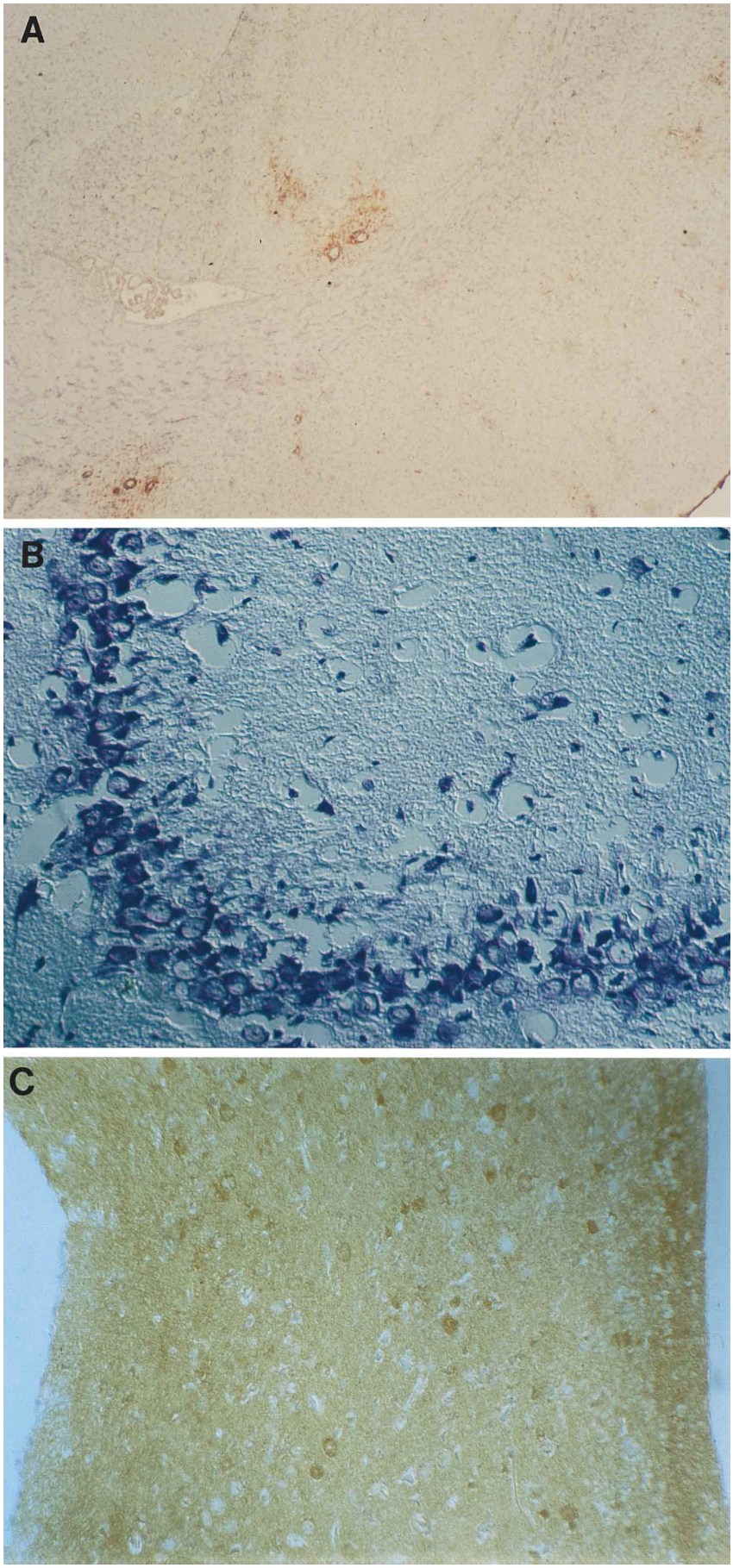


FIG. 4. (A) Adoptive transfer of low numbers of brain lymphocytes (BrLy) isolated from BDV-infected rats at day 13 or 21. (B) Severity of neurological symptoms. (C) Decrease of body weight.

without transfer showed a rather late transient reduction of body weight, leveling back to the original body weight, without showing clinical symptoms (Fig. 4B and C). In another set of experiments, as few as 5×10^5 total cells (7×10^4 $CD8^+$ cells) caused disease symptoms at 13 days after i.v. transfer. These recipients also had histological and immunohistological alterations comparable to those mentioned above (data not shown).

Encephalitis and neurodegeneration after adoptive transfer of brain lymphocytes. In another experiment, rats receiving 7×10^6 brain-isolated cells were killed at the time of onset of clinical disease (day 7) and at days 10 and 28 and were examined histologically, immunohistologically, and virologically. As early as 7 days p.t., an encephalitic reaction was found (score of 1.5), which had increased to maximal inflammation (score of 3.0) already by day 10. Most intriguing was the finding of an inflammatory reaction with the beginning of migration of $CD8^+$ T lymphocytes from perivascular accumulations of inflammatory cells into the brain parenchyma (Fig. 5A). $CD4^+$ T cells remained predominantly in the perivascular location (data not shown). Thus, the distribution of $CD4^+$ and $CD8^+$ cells after passive transfer exactly reflects the situation seen in experimentally BDV-infected rats, as reported earlier (2). Consequently, the first signs of spongiform degeneration of the neuropil, comprising pericapillary and astrocytic edemas as well as nerve cell degenerations, were already seen between days 7 and 10 p.t. (Fig. 5B), resulting finally in brain atrophy. Cortical brain atrophy, which can be usually observed in BDV-infected rats after day 50 to 70 p.i., was severe already at day 28 (Fig. 5C). In recipient rats receiving lower numbers of brain lymphocytes (2.5×10^6), the onset of degenerative alterations was delayed, and so were the kinetics of CD8 and perforin mRNAs. RT-PCR analyses of CD8 and perforin mRNAs in recipients of day 21 brain lymphocytes revealed the presence of both mRNAs at day 16 p.i. at the latest but not at day 11 or 35 p.i. The distribution of BDV-specific antigen (data not shown) and virus titers in the brains of all of these recipients (10^4 $TCID_{50}$ at day 7 and 10^6 $TCID_{50}$ at day 28) were comparable to those for control rats.

Low pathogenic potential in cervical lymph node and spleen lymphocytes. Despite the absence of typical lymphatic chan-


A
B
C

nels, proteins can pass from the brain into the peripheral lymphatics. Since in an earlier study (22) we were unable to detect cytolytic activity in lymphocytes isolated from either peripheral blood, spleen, or abdominal lymph nodes, we have included the testing of cervical lymph nodes in the present study. Upon testing of isolated lymphocytes without in vitro cultivation, we were not able to detect cytolytic activity in vitro (data not shown). Adoptive transfers of freshly isolated lymphocytes into immunosuppressed BDV-infected recipients did not induce BD-specific symptoms. However, high cell numbers (5×10^7) but not lower cell numbers (5×10^6) caused unspecific clinical symptoms (mainly passiveness and lack of food uptake and, as a consequence, loss of body weight) 5 days after transfer (data not shown). Histologically, despite a few perivascular infiltrations in the meninx, no inflammatory reactions were seen in the brain, whereas in the lungs, the intestines, and abdominal lymph nodes, massive cellular accumulations of significant numbers of $CD4^+$ cells but only very few $CD8^+$ cells were found (data not shown). However, after secondary in vitro restimulation with purified BDV-specific antigen or recombinant proteins, cervical lymph node lymphocytes caused BD-specific neurological symptoms upon adoptive transfer (Fig. 6). In this case, the first inflammatory reactions were observed after 8 to 9 days p.t., but neuronal necrosis was seen only when the remaining rats were killed between days 14 and 21 p.t. The same pattern was observed when secondarily restimulated spleen cells were transferred.

DISCUSSION

Adoptive transfer of lymphocytes isolated from the brains of diseased Lewis rats into healthy immunosuppressed BDV-infected recipients resulted in brain lesions that reflect early morphological changes after experimental infection. These early changes comprise pericapillary and intracellular astrocytic edemas, vacuolation of the neuropil, and degeneration of neurons. The presence of considerable numbers of $CD8^+$ T cells and only very few $CD4^+$ T cells in the brain parenchyma near the lesions suggests that $CD8^+$ T cells are of crucial importance in the destruction of virus-infected brain cells. This view is supported by the fact that MHC class I-restricted cytotoxicity is found in brain lymphocyte preparations from days 13 and 21 p.i. These lymphocyte preparations were able to cause severe brain cell degeneration and BD after adoptive transfer. Most intriguingly, lymphocytes from days 13 and 21 were able to cause necrobiotic changes, and already at 28 days p.t. brain atrophy could be observed, which can never be seen in experimentally infected rats before day 50 p.i. The destruction of the brain appears to be correlated with the presence of perforin mRNA, the major effector molecule in CD8-mediated lysis of target cells (20). Whereas the first $CD8^+$ T cells could be found between days 9 and 11 p.i., perforin was detected only after day 13 p.i. Therefore, lymphocytes isolated after day 13 p.i. represent effector cells with regard to their MHC class I-restricted lysis of target cells in vitro and their ability to produce perforin. Most of the experiments described here were carried out with lymphocytes from day 21, merely due to the higher numbers that could be recovered compared to cell numbers recovered at day 13 p.i. In contrast to cells obtained early after infection, the vast majority of brain lymphocyte preparations isolated from individual rats at day 35 exhibited no cytotoxic activity, and likewise, no upregulation of perforin mRNA was found. However, in a very few rats low cytotoxic activity was still found on day 35, providing evidence for a loss of lymphocytic cytotoxic activity around this time. Our experiments might be interpreted to indicate that $CD8^+$ T cells enter the brain with little or no cytotoxic activity, because upon immunohistological examination and detection of mRNA by RT-PCR, $CD8^+$ cells can be found after day 9 or 11, whereas mRNA upregulation of perforin was found only after day 11. The presence of perforin correlates with the first degenerations in brain cells, which can also be seen after day 11 in BDV-infected rats (2). We therefore propose that BDV-specific cytotoxic T lymphocytes are primed outside the central nervous system, as has been suggested earlier (36), and thereafter gain access to the brain, where the synthesis of perforin is induced on the site. However, the possibility of local priming of $CD8^+$ T cells but not of $CD4^+$ T cells in the brain has been demonstrated (29) and represents an alternative explanation for our virus-induced model of brain disease. The expression of perforin has to be restricted to $CD8^+$ T cells, since we have found no evidence for the presence and activity of NK cells (22). Th1 cells, which have been proposed to play an important role in BD (24), do not lyse their targets via the perforin pathway, whereas Th2 cells do (17). However, Th1 cells are capable of lysing target cells by other mechanisms (17), one of which might be the Fas-dependent pathway (15, 18, 38). Indeed, whereas perforin-mediated lysis of target cells in CD8 killing has been demonstrated and established (13, 20), a second pathway via Fas and its ligand might be operative under certain circumstances and be dependent on the expression of Fas by the target cell (9, 12, 31). Due to this consideration and due to findings by various groups which indicate that the Fas-dependent pathway plays a more prominent role in CD4-mediated lysis than in CD8-mediated killing, we cannot exclude lytic activity of $CD4^+$ T cells; however, the importance of a Fas-mediated pathway in viral infections has not yet been established even for CD8-mediated cytotoxicity (14).

Because of several reasons, we think that a direct cytotoxic activity from $CD4^+$ T cells does not play an important role in BD. First, only very few, if any, $CD4^+$ T cells can be found in the brain parenchyma, where the destruction of cells occurs, whereas $CD8^+$ T cells are numerous; $CD4^+$ T cells are found predominantly in perivascular cuffs (2, 7). The same was true for the adoptive transfer experiments reported here. Brain lymphocytes which exerted MHC class-I restricted cytolysis in vitro were adoptively transferred in immunosuppressed, persistently infected recipients. In these recipients $CD8^+$ T cells were localized immunohistochemically in the brain tissue undergoing spongiform degeneration. Second, we have found no convincing evidence for an MHC class II-restricted lysis (this report and reference 22). Third, adoptive transfer of a BDV-specific $CD4^+$-T-cell line that showed no cytotoxic activity in vitro was still able to induce BD in recipients; however, in the

FIG. 5. Histological and immunohistological findings in brains of recipients rats. (A) Presence of $CD8^+$ T cells in the cerebral cortex and the CA3 area of the hippocampus in a cyclophosphamide-immunosuppressed recipient rat persistently infected with BDV 7 days after adoptive transfer of BDV-specific brain-derived lymphocytes. T cells are predominantly located perivascularly but have started to infiltrate the brain parenchyma. Magnification, ×40. (B) Sector CA3 of the hippocampus of a recipient rat at day 10 after transfer. Severe pericapillary and cellular edemas comprising spongiform degeneration of the neuropil and nerve cell necroses are seen. Magnification, ×400. (C) Marked cortical atrophy in a recipient rat at day 28 after transfer. Note the reduction of the cortical diameter and the dramatic numerical loss of neurons, about 30% of which express BDV antigen p40. Lymphocytes are no longer present. Magnification, ×180. Staining was with MAb OX-8 (A), MAb Bo-18 (C), and Nissl stain (B).

A

group	Ly	total number	no. of recipients
1	CLN day 13	$2x10^6$	2
2	CLN day 21	$2x10^6$	2
3	Spleen day 13	$2x10^6$	2
4	-	-	2

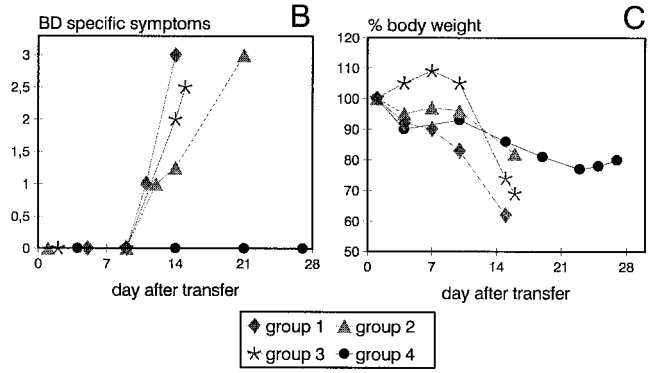


FIG. 6. (A) Adoptive transfer of cervical lymphnode cells (CLN) and spleen cells after secondary in vitro restimulation with BDV-specific antigen. (B) Severity of clinical symptoms. (C) Decrease of body weight.

brains of recipient rats, in addition to $CD4^+$ T cells which again accumulated in perivascular locations, numerous $CD8^+$ T cells were found in the parenchyma (23). Furthermore, recipient rats in which $CD8^+$ T cells had been blocked by MAbs (35) did not succumb to the disease after adoptive transfer (23). The presence of $CD8^+$ T cells was also verified by immunohistological examination of rats that had received cervical lymph nodes or spleen cells after secondary in vitro restimulation. In this case, degenerative alterations were found despite the seeming absence of cytotoxic activity in transferred cells. The lack of cytotoxic activity in the periphery has been reported before for BD and a variety of other viral infections (22). A possible explanation for this finding might be that T-helper-cell activity can be restimulated in vitro but that $CD8^+$ T cells from rats do not proliferate readily in vitro, as is common knowledge. The transfer of T helper cells, however, would result in the recruitment of inflammatory cells, including $CD8^+$ T cells, as has been shown previously by our group using a BDV-specific T-helper-cell line (23). This would also explain the delay of degenerative changes in the brain after transfer of in vitro-restimulated cervical lymph node and spleen cells. The data presented here, together with our earlier work, provides evidence that cytotoxic T cells strike first and initiate cellular destruction in the brain. The adoptive transfer experiments reported here support this view. Thereafter, probably several other pathways contribute to the deleterious immune response which finally results in the destruction of brain tissue and leads to chronic debility and dementia (21). Since microglia cells can kill infected neighboring cells directly (8) and also produce cytokines and radicals, we cannot exclude the possibility that activated microglia cells join T cells in the process of tissue destruction by their abundance and probably unrestrained activity during an ongoing inflammatory reaction in the brain. The number of mediators which are involved in immunopathological reactions during BD and other inflammatory brain diseases is steadily increasing, and insight into their immunoregulatory effects will also add to our understanding of mechanisms that act in addition to and/or as a consequence of cytotoxic-T-cell responses in this model infection of degenerative brain disease.

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