JOURNAL OF VIROLOGY, Feb. 1995, p. 896–903
0022-538X/95/$04.00+0

Vol. 69, No. 2

Immunopathogenic Role of T-Cell Subsets in Borna Disease Virus-Induced Progressive Encephalitis

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Received 1 August 1994/Accepted 10 November 1994

Borna disease is an immunopathological virus-induced encephalopathy comprising severe inflammation and degenerative brain cell lesions which results in organ atrophy and chronic debility in rats. $CD4^+$ and $CD8^+$ T cells have been reported to be involved in the development of this disease of the central nervous system. A virus-specific homogeneous T-cell line, established in vitro after immunization of rats with the recombinant 24-kDa virus-specific protein, showed antigen-specific proliferation in the presence of the 24-kDa but not the 38-kDa Borna disease virus-specific protein, another major virus-specific antigen. This T-cell line, P205, was found to exhibit characteristics of a T-helper cell: $CD4^+$ $CD8^-$ IL-2^- IL-4^- IFN-γ^+ IL-6^+ IL-10^+. Furthermore, this T-cell line expressed the α/β T-cell receptor and the $\alpha 4$ integrin (VLA-4). Adoptive transfer of this helper cell resulted in an increase of antibody titers and two different types of disease in virus-infected rats after cyclophosphamide-induced immunosuppression. (i) Rats receiving T cells between 10 and 18 days after treatment with cyclophosphamide showed an acute lymphoproliferative disease in the gut and lungs within 9 days after adoptive transfer and died. (ii) Passive transfer within the first 5 days after immunosuppressive treatment resulted in typical Borna disease associated with neurological symptoms such as ataxia and paresis starting 14 to 16 days after transfer. Immunohistological analysis of the brains of rats with Borna disease uniformly revealed the presence of $CD8^+$ T cells in encephalitic lesions in addition to $CD4^+$ cells that were found in the brains of recipients of the virus-specific $CD4^+$ T-cell line, irrespective of whether neurological symptoms developed or not. However, recipient rats treated with antibodies against $CD8^+$ T cells developed neither encephalitis nor disease. Therefore, $CD4^+$ T cells appear to accumulate in the brain and cause perivascular inflammatory lesions which alone obviously do not cause disease. In contrast, the presence of $CD8^+$ cells apparently directly correlates with the development of neurological symptoms.

After infection of rats with Borna disease virus (BDV), a single-stranded RNA virus (9, 14), a central nervous system disease develops that finally results in brain atrophy and chronic debility (7, 8, 23, 32). The virus, which is tightly cell associated, lacks apparent cytopathogenicity in vitro (20), and there is only circumstantial evidence of in vivo cytopathogenicity (11). The virus replicates preferentially in cells derived from the neural crest such as neurons, astrocytes, and ependymal cells (11, 12, 15, 32); however, evidence of infection and an inflammatory response in the peripheral and autonomic nervous system has also recently been demonstrated (10, 12). The disease has been shown to be based on an immunopathological reaction (reviewed in reference 44) in which the cellular immune response, but not an antibody-mediated reaction to virus-specific antigens, results in severe encephalitis (21, 32, 48).

The original finding that $CD4^+$ T cells play a role in the development of the encephalitis was based on experiments with a $CD4^+$ T-cell line specific for a 38- to 39-kDa virus-specific protein (38, 39). This T-cell line showed some notable characteristics: adoptive transfer into cyclophosphamide (CY)-treated recipients between days 10 and 15 after immunosuppression resulted in death, or animals had to be killed in a moribund condition as early as 5 to 10 days after receiving the T cells. Furthermore, this cell line was found to lyse virus-infected target cells in vitro in a major histocompatibility complex (MHC) class II-restricted manner (37). More recent studies have revealed the involvement of $CD8^+$ T cells in Borna disease (BD) in rats (8, 35, 46, 47).

In the present report, we present for the first time the complete phenotypical and biological characterization of a BDV-specific $CD4^+$ T-cell line. The adoptive transfer of this 24 kDa protein-specific T cell line into virus-infected, CY-treated recipients resulted either in neurological disease with BD symptoms or in rapid death, dependent on whether the transfer occurred early or late after infection and immunosuppression, respectively. Rats that were treated with monoclonal antibodies (MAb) against $CD8^+$ cells and received the 24-kDa BDV protein-specific $CD4^+$ T-cell line did not show any clinical symptoms. These results support the view that both $CD4^+$ and $CD8^+$ T cells are operative in the pathogenic mechanism of BD and provide evidence that $CD8^+$ T cells are indispensable for BDV-induced immunopathology in the brain.

MATERIALS AND METHODS

Virus and experimental animals. Giessen strain He/80 of BDV was used throughout all studies. Five-week-old female Lewis rats were obtained from the Zentralinstitut für Versuchstierzucht, Hannover, Germany, and inoculated intracerebrally in the left hemisphere with 5×10^3 50% tissue culture-infective doses of BDV.

Propagation of the T-cell line. Lewis rats (8 to 10 weeks old) were immunized with 50 μg of a recombinant BDV-specific 24-kDa antigen (r24kd; reference 53) (kindly provided by H. Niemann, Tübingen, Germany) into both hind footpads. Twelve days later, the animals were anesthetized and popliteal lymph nodes were collected. Lymph nodes were mixed, and lymphocytes were separated by Lympholyte R (Cedarlane, Hornsby, Ontario, Canada) gradient centrifugation. In a secondary in vitro restimulation, 5×10^5 cells were cultured in the presence of 30 μg of the r24kd antigen per ml together with the same number of irradiated syngeneic thymocytes. Thereafter, 5×10^5 lymphocytes were restimulated again with 5×10^6 irradiated syngeneic thymocytes and 30 μg of the r24kd antigen per

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ml for 4 days. Finally, the lymphocytes were cultured with 10% interleukin 2 (IL-2) supernatant-containing medium for 5 to 6 days before further restimulation with a specific antigen and syngeneic thymocytes, followed by cultivation again in the presence of IL-2. A BDV-specific antigen was also purified from the brains of infected rats by affinity chromatography by employing MAb (52) revealing the 38- and 24-kDa proteins.

Clinical evaluation. All experimental animals were examined daily and weighed, and disease symptoms were scored by two independent observers on an arbitrary scale of 0 to 3 based on the general state of health and the appearance of neurologic symptoms (scores: 1, slight uncoordination and fearfulness; 2, distinct ataxia or slight paresis; 3, paresis or paralysis). Body weight on the day of infection was defined as 100%, and the percentage of weight change was calculated.

Proliferation assay. T cells (5×10^4) were cultured in the presence of 5×10^5 irradiated syngeneic thymocytes and in the presence of 30 μg of the r24kd or r38kd (provided by W. I. Lipkin, Irvine, Calif.) BDV-specific protein per ml, influenza virus hemagglutinin, or vaccinia virus proteins or without antigen in flat-bottom 96-well microtiter plates for 60 h. Thereafter, 0.2 μCi of [^{3}H]thymidine per well was added and after an additional 12 h the cells were collected and [^{3}H]thymidine incorporation was measured.

Reverse transcription-PCR analysis of lymphokine mRNA levels. For reverse transcription-PCR, total cellular RNA was isolated after CsCl gradient centrifugation from 10^7 cells of the r24kd-specific T-cell line and from several clones derived from this line. Magnetic beads (Dynabeads; Dianova, Hamburg, Germany) were used to separate the mRNA from the total cellular RNA as described by the manufacturer. RNA was reverse transcribed by using an 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 volume containing 100 ng of each oligonucleotide primer per μl; 10 mM each dATP, dTTP, dGTP, and dCTP (Pharmacia, Freiburg, Germany); and 10 μl of a 10× buffer containing 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 with oligomers that amplify segments of 410 (IL-2), 378 (IL-4), 636 (IL-6), 284 (IL-10), 413 (gamma interferon [IFN-γ]), and 607 (β-actin) bp. Ten microliters was loaded onto a 2% agarose minigel and visualized by ethidium bromide staining.

Alternatively, nested reverse transcription-PCR was done for IL-2 and IL-4 to enhance the sensitivity of the amplification products (41). 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 in the second PCR two nested primers were used which were internal to the first primer pair. The larger fragment produced by the first reaction was used as the template for the second PCR.

Hot-start PCR was performed in all cases. In this procedure, the polymerase was added after denaturation and preheating of the sample to 80°C to avoid nonspecific primer hybridization.

The following primers were used as described by Shankar et al. (40): external IL-2 sense, 5′-CATGTACAGCATGCAGCTCGCATCC-3′; external IL-2 antisense, 5′-CCACCACAGTTGCTGGCTCATCATC-3′; internal IL-2 sense, 5′-CAGGTGCTCCTGAGAGGGATCG-3′; internal IL-2 antisense, 5′-GAGCCC TTGGGGCTTACAAAAAG-3′; external IL-4 sense, 5′-TGATGGGTCTCAG CCCCCACCTTGC-3′; external IL-4 antisense, 5′-CTTTCAGTGTTGTGAGC GTGGACTC-3′; internal IL-4 sense, 5′-AACACCACGGAGAACGAGCTCA TC-3′; internal IL-4 antisense, 5′-AGTGAGTTCAGACCGCTGACACCT-3′; IL-6 sense, 5′-ATGAAGTTTCTCTCCGCAAGAGACTTCCAGCCAG-3′; IL-6 antisense, 5′-CTAGGTTTGCCGAGTAGACCTCATAGTGACC-3′ (the IL-10 primer was a gift from Ian Lipkin, University of California, Irvine [unpublished data]); β-actin sense, 5′-ATGCCATCCTGCGTCTGGACCTGGC-3′; β-actin antisense, 5′-AGCATTTGCGGTGCACGATGGAGGG-3′; IFN-γ sense, 5′-GTTACTGCCAAGGCACACTCATTGAAAGCC-3′; IFN-γ antisense, 5′-TCAGCACCGACTCCTTTTCCGCTTCCTTAGGC-3′.

Immunosuppression with CY or anti-T-cell MAb. One day after intracerebral BDV infection, the animals received 160 mg of CY per kg intraperitoneally (32). Alternatively, thymectomized rats were treated with 2 mg of purified mouse MAb directed against rat CD8$^+$ T cells (OX-8) or directed against all T cells (OX-52) 1 day before and 1 day after infection (47). After immunosuppression or antibody treatment, infected animals developed no signs of BD.

Adoptive transfer of the BDV-specific T-cell line. For adoptive-transfer studies, 10^5 to 10^7 activated cells of BDV-specific T-cell line P205 were injected intravenously after restimulation with the specific antigen and after 1 day of incubation in IL-2-containing medium at different time points after BDV infection.

Cytotoxicity assay. ^{51}Cr release assays were performed as previously described (35). Briefly, persistently infected syngeneic skin fibroblasts and an astrocytic cell line (F10; kindly provided by H. Wekerle, Munich, Germany) were treated with rat IFN-γ for 72 h to induce MHC class II expression. Target cells were labeled with 0.2 mCi of ^{51}Cr per 10^7 cells at 37°C for 1 h. P205 effector T cells and target cells were incubated at an effector-to-target cell ratio of 30:1 and at threefold dilutions of the original concentration of effector cells. After various periods of time (6 to 20 h), 50-μl samples were collected and counted in a gamma counter.

TABLE 1. Antigen-specific proliferation of CD4$^+$ T-cell line P205[a]

T-cell line	cpm of [^{3}H]thymidine incorporation (SI[b])					
	BDV antigen	r24kd	r38/39kd	HA[c]	VVP[d]	NS[e]
P205	16,663 (19)	22,948 (25)	2,294 (3)	1,554 (2)	1,062 (1)	877
Control	3,087 (21)	1,994 (14)	1,617 (11)	ND	ND	147

[a] Proliferation of P205 cells was tested after various restimulation cycles in the presence of a BDV-specific or irrelevant antigen. For details, see Materials and Methods. The control was a T-cell line induced by a mixture of native BDV-specific proteins and cultured in the presence of the same antigens. ND, not done.
[b] SI, stimulation index.
[c] HA, influenza virus type A hemagglutinin.
[d] VVP, vaccinia virus proteins.
[e] NS, no stimulation.

BDV-specific antibody measurement. BDV-specific antibodies were detected by an enzyme-linked immunosorbent assay method as previously described (48).

Histology and immunohistochemistry. Animals were killed at different time points after BDV infection. Brain tissue was either frozen in isopentane at −150°C or fixed in buffered paraformaldehyde. All tissue sections were stained with hematoxylin and eosin and with cresyl violet as described by Nissl. Encephalitic infiltrates were scored on an arbitrary scale of 0 to 3 based on the number of infiltrates per section and the number of cell layers in each infiltrate (scores: 1, up to 5 small infiltrates per section; 2, more than 5 small infiltrates per section or more than 3 infiltrates with multiple layers; 3, more than 10 small infiltrates or more than 5 infiltrates with multiple layers). Immunohistochemical analysis was carried out on cryostat sections to detect the presence of lymphocyte subsets and macrophages, as well as MHC antigens, IFN-γ, and tumor necrosis factor. The following MAbs (Serotec) were used: OX-8 for CD8$^+$ T cells, W3/25 and OX-38 for CD4$^+$ T cells, and ED1 for macrophages. The percentages of positive cells reactive with these MAb were determined by visual counting (8, 46).

Cytofluorometry. Unstained and stained P205 T cells were scanned on an Epics Elite laser flow cytometer (Coulter Electronics, Hialeah, Fla.). During data acquisition, the T-cell population was gated to exclude debris and 10^4 cells were counted per sample. Cells were incubated with various fluorescein isothiocyanate-conjugated MAb 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 P12520 (VLA-4) and R73 (α/β T-cell receptor) (Dianova).

RESULTS

Induction and characterization of a BDV-specific 24-kDa T-cell line. Since the recombinant 24-kDa BDV-specific protein (r24kd) was used to induce T cells, we retested the immunologic relationship between the natural and recombinant 24-kDa proteins (53). Western blot (immunoblot) analysis and immunoprecipitation revealed the reactivity of both proteins with polyclonal BDV-specific antisera and MAb induced by either the native or the recombinant 24-kDa protein (data not shown). Popliteal lymph nodes obtained from rats immunized with the r24kd protein were removed, and lymphocytes were isolated and propagated in vitro. Numerous cycles of antigen-specific stimulation in the presence of irradiated syngeneic thymocytes and cultivation in IL-2-containing medium in the absence of virus-specific protein were carried out. To establish the antigen specificity of the T cells, proliferation assays were performed with native BDV-specific antigen purified either from the brains of BDV-infected rats by affinity chromatography or with recombinant proteins (Table 1). The results revealed antigen specificity for the r24kd protein compared with a T-cell line specific for the 38- or 39-kDa BDV-specific protein.

Furthermore, cultured r24kd-specific lymphocytes were analyzed for phenotypic markers in cytofluorometric assays. As shown in Fig. 1, the phenotype of the 24 kDa-specific lymphocytes is typical of CD4$^+$ T cells: W3/13$^+$ W3/25$^+$ OX-8$^-$ OX-33$^-$. The cell line was also found to carry the α/β T-cell re-

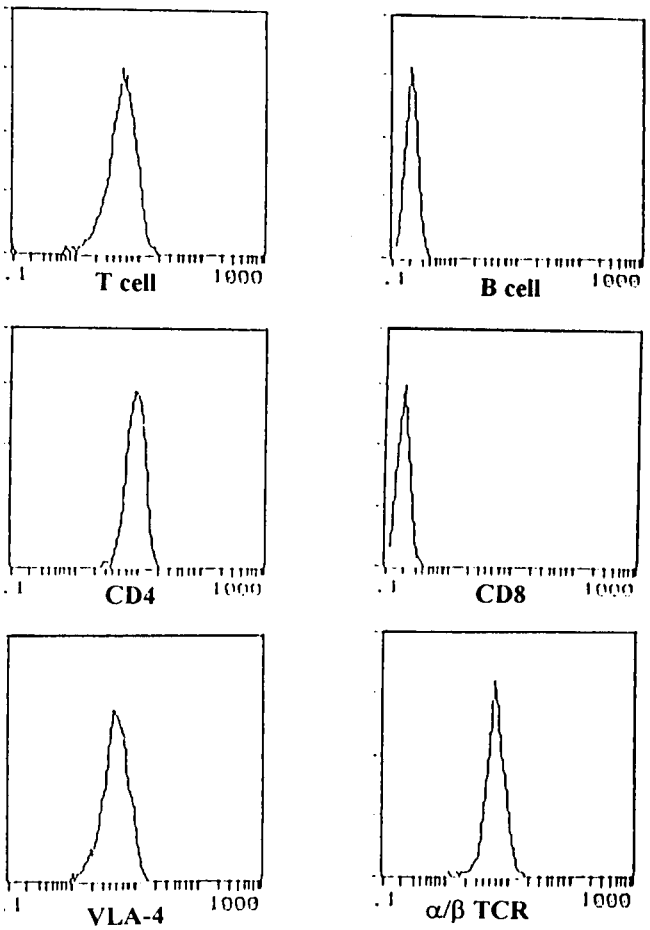

FIG. 1. Cytofluorographic histograms of T-cell line P205. The MAb used were W3/13 (T cells), OX-33 (B cells), W3/25 (CD4), OX-8 (CD8), P12520 (VLA-4), and R73 (α/β T-cell receptor). For details, see Materials and Methods.

ceptor (MAb R73; reference 25) and express the VLA-4 (α4 integrin) molecule. This 24-kDa BDV-specific T-cell line is called P205.

To establish the cytokine profile, 10^7 cultured lymphocytes were lysed and RNA was extracted for reverse transcription-PCR. As shown in Fig. 2, PCR analyses revealed the presence

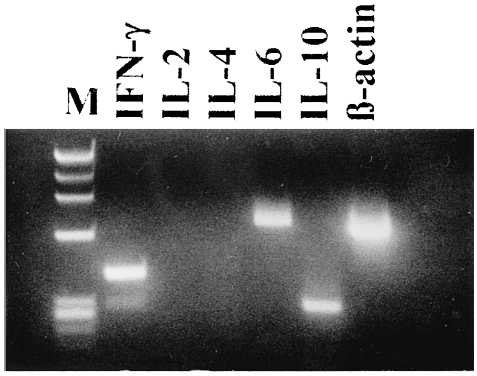

FIG. 2. PCR analyses of different cytokine mRNAs of $CD4^+$ 24-kDa-specific T-cell line P205. Lane M, molecular size markers (ϕX174 digested with *Hae*III). For further details, see Materials and Methods.

TABLE 2. Effect of adoptive transfer of the P205 BDV-specific T-cell line on antiviral antibody synthesis[a]

Expt	CY treatment	Transfer	BDV-specific antibody titer		
			Day 10 p.i.	Day 13 p.i.	Day 24 p.i.
Control	−	−	<1:40	1:80	>1:5,120
1A	−	+	1:160	1:320	>1:5,120
2A	−	+	1:320	1:1,280	>1:5,120
3A	−	+	1:40	1:80	ND
Control	+	−	<1:40	<1:40	<1:40
1B	+	+	<1:40	<1:40	1:80
2B	+	+	<1:40	<1:40	1:160

[a] BDV-infected, untreated (CY^-) and BDV-infected, CY-treated, immunosuppressed (CY^+) rats received 2.5×10^6 (experiments 1A and 2A and 1B and 2B) or 2.5×10^5 (experiment 3A). P205 $CD4^+$ T cells, and serum antibody titers were measured at different time points with an enzyme-linked immunosorbent assay. The antibody titers given represent the means of three animals. Titer variations never exceeded one level. ND, not done.

of mRNAs specific for IL-6, IL-10, and IFN-γ, whereas no reaction was found with oligonucleotide primers for IL-2 and IL-4.

The cytotoxic activity of the P205 cell line was tested on syngeneic BDV-infected fibroblasts and astrocytes. Infected astrocyte target cells were pretreated with IFN-γ for 72 h to induce MHC class II antigen expression (59). Cytofluorometric analysis and indirect immunofluorescence revealed the presence of MHC class II antigen on BDV-infected astrocytic cells but not on fibroblasts (data not shown) as described earlier (35, 37). Neither MHC class I-positive fibroblast nor MHC class II-positive astrocyte target cells were lysed; in addition, P205 cells did not exert natural killer activity since NK-sensitive YAC-1 target cells were not lysed (data not shown).

T-helper activity of BDV-specific $CD4^+$ T-cell line P205. To determine whether the P205 cell line exerts helper activity in vivo, adoptive-transfer experiments were carried out with BDV-infected rats. Activated P205 cells were adoptively transferred into BDV-infected, untreated rats or infected and then CY-treated recipients (Table 2).

BDV-infected, not immunosuppressed recipients of 2.5×10^6 P205 T cells showed antiviral antibodies as early as 5 days posttransfer (p.t.), i.e., 10 days postinfection (p.i.), whereas infected but otherwise untreated control animals had no detectable antibodies at this time point (Table 2, experiments 1A and 2A). At day 8 p.t. (day 13 p.i.), the antibody titers of rats having received P205 cells had further increased compared with those of infected, untreated rats, in which low antibody titers were detected only after day 12 p.i. (Table 2, experiments 1A to 3A). Antibody titers directly correlated with the number of cells transferred; transfer of 2.5×10^6 cells (experiments 1A and 2A) resulted in two- to fourfold higher antibody titers than did transfer of 2.5×10^5 cells (experiment 3A).

Because of the immunosuppressive effect of CY, animals treated with CY 1 day after BDV infection did not synthesize detectable antiviral antibodies (Table 2, experiments 1B and 2B). After passive transfer of P205 T cells, however, antibodies specific for BDV were detected at day 19 p.t. (day 24 p.i.) (Table 2, experiments 1B and 2B). Thus, rat T-cell line P205 shows characteristics of a functional T-helper cell line.

Induction of immunopathological disease after adoptive transfer of BDV-specific $CD4^+$ T-cell line P205. Passive transfer of P205 T cells into normal or CY-treated, uninfected recipients resulted in neither pathological brain alterations nor clinical signs, providing evidence that this T-cell line is not encephalitogenic (data not shown).

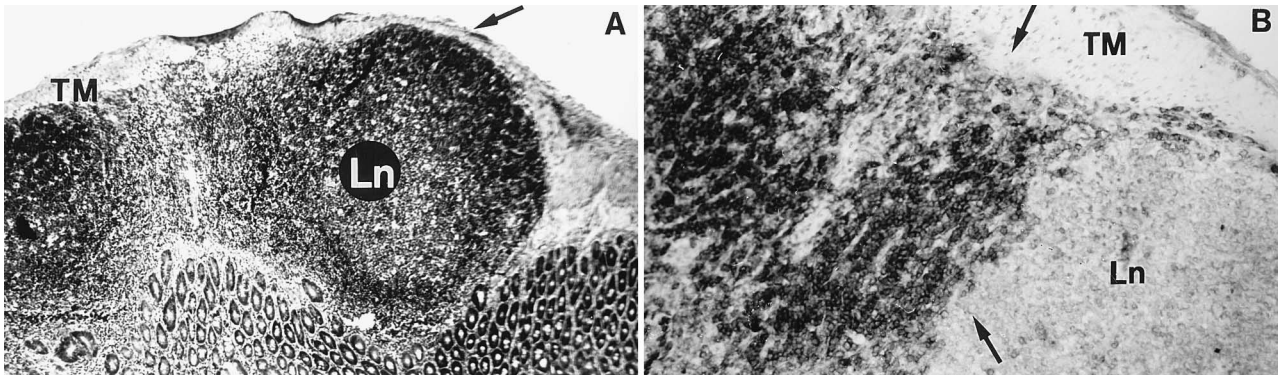

FIG. 3. Severe lymphocellular proliferation in Peyer's patches and diffuse proliferation within the intestinal wall in a BDV-infected immunosuppressed rat subjected to adoptive transfer of $CD4^+$ T-cell line P205 at day 10 after CY treatment. (A) Lymphocytic infiltration extends from the epithelial layer (lamina epithelialis) into the muscular layer (tunica muscularis), which is markedly reduced in size (arrow). Staining was done with hematoxylin and eosin. Magnification, ×50. (B) Immunohistochemical analysis of a corresponding slide with MAb OX-38 showing that most of the diffusely infiltrating cells outside the nodule are CD4 positive (arrows). TM, tunica muscularis; Ln, solitary lymph nodule. Immunohistochemical staining was done by the ABC method. Magnification, ×80.

Transfer of activated P205 T cells into BDV-infected, immunosuppressed rats resulted in two different pattern of disease dependent on the time of transfer relative to the time point after CY treatment.

(i) Late transfer. Late transfer of 10^6 to 10^7 P205 cells on day 10 or 15 after immunosuppression resulted in a drastic weight loss starting immediately after injection (Fig. 4). All rats receiving 10^7 or 10^6 P205 cells died within 7 to 9 days after transfer without developing neurological symptoms. Quantitation of brain lymphocytes was not done because of the low encephalitis scores, irrespective of the fact that both T-cell subpopulations were identified immunohistochemically. In the periphery, i.e., the lungs and intestines, most (>70%) of the lymphocytes were $CD4^+$ (Table 3).

Histological examination of the brains of BDV-infected, immunosuppressed recipients of P205 T cells revealed only moderate encephalitis, which did not correlate with their general bad health. Macroscopically, the entire intestine, including the mesenteric lymph nodes, showed signs of severe lymphoproliferative disease, i.e., many nodules in the intestinal wall and the regional lymph nodes. Histologically, a massive lymphocytic infiltration was found in the intestinal wall, in mesenteric lymph nodes, in Peyer's patches, and in the lungs of BDV-infected rats having received the BDV-specific T-cell line later than 10 days after immunosuppression (Fig. 3A). Immunohistological characterization of infiltrating cells revealed the majority of the lymphocytes diffusely infiltrating the intestinal wall to be CD4 positive (Fig. 3B). Animals receiving 10^5 cells and uninfected rats receiving the highest number of $CD4^+$ T cells showed normal weight gain and no clinical symptoms throughout an observation period of 100 days (data not shown).

(ii) Early transfer. Early transfer of P205 T cells 4 days after immunosuppression resulted in disease symptoms comparable to those of infected but otherwise untreated rats. By day 12 p.t., recipient rats showed moderate weight loss (Fig. 4), ruffled fur, and hunched backs. Neurological symptoms such as slight ataxia were first detectable at day 14 p.t. and increased during the next few days, resulting in paresis and, in some cases, paralysis by day 18 or 19 p.t. (Table 4). One rat died on day 19 p.t., but otherwise all other animals were killed on the same day because of their bad health. Macroscopical and histological examination of lymph nodes, intestines, and lungs did not reveal any significant cellular infiltration. In the brain, however, severe encephalitic reactions on day 14 that increased until day 19 were observed (Fig. 5).

Immunohistologically, more than 50% of the inflammatory cells were $CD4^+$ T cells and about 20% of T cells in perivascular infiltrations and about 30 to 40% of T cells in parenchymal infiltrations were CD8 positive (Fig. 5). BDV-infected, CY-treated controls which had not received T cells showed neither encephalitic lesions nor disease (data not shown).

Absence of immunopathological disease after transfer of cell line P205 into anti-T-cell antibody-treated recipients. Experiments similar to those described above were carried out with thymectomized rats which had been treated with MAb OX-8 (directed against $CD8^+$ cells) or OX-52 (directed against all T cells). Rats treated in this way have been shown to stay healthy after BDV infection (47). Adoptive transfer of as many as 5 ×

TABLE 3. Effect of transfer of P205 $CD4^+$ T-cell line into BDV-infected recipients late after CY immunosuppression[a]

No. of cells transferred	Outcome	Day p.t.	Encephalitis		T cells present in:		Body wt change (%)
			No. of rats with encephalitis/total	Mean score ± SEM	Brain[b]	Lungs, intestines[c]	
10^7	6 of 6 dead	7	3/6	0.4 ± 0.5[d]	CD4, CD8	CD4	−29
10^6	6 of 6 dead	9	4/6	0.5 ± 0.8[e]	CD4, CD8	CD4	−28
10^5	6 of 6 killed	28	2/6	0.3 ± 0.3[f]	CD4, CD8	CD4	−4

[a] Recipient rats were subjected to adoptive transfer of the indicated numbers of P205 T cells on day 10 p.i., i.e., day 9 after CY immunosuppression.
[b] Quantitation of brain lymphocytes was not done because of the low encephalitis scores.
[c] More than 70% of lymphocytes were $CD4^+$; $CD8^+$ cells were not found.
[d] The individual scores were 0, 0, 0, 0.5, 0.5, and 1.5.
[e] The individual scores were 0, 0, 0, 0, 1.5, and 1.5.
[f] The individual scores were 0, 0, 0.5, 0.5, 0.5, and 0.5.

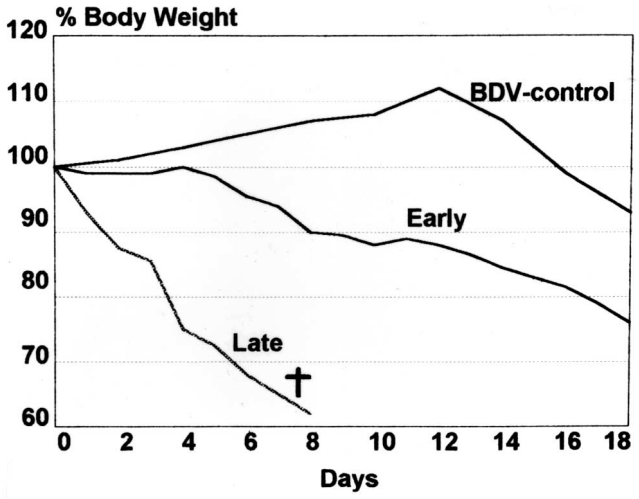

FIG. 4. Growth curves of BDV-infected, CY-treated rats receiving adoptive transfer of P205 T cells late (Late) or early (Early) after immunosuppression and of untreated, BDV-infected control animals (BDV-control). Day zero was the day of cell transfer (Early and Late) or BDV infection (BDV-control). †, death.

10^6 $CD4^+$ P205 T cells, which caused encephalitis and BD in CY-treated rats, did not cause weight loss, clinical symptoms, or inflammation of the brain (Table 5). In contrast, antibody-treated rats receiving immune spleen cells from BDV-infected donors showed BD symptoms and had encephalitis.

Histological examination revealed the presence of few inflammatory cells in the brain, as well as in the lungs and liver (data not shown). A slight cellular infiltration was also observed in OX-8-treated control rats (Table 5) which had not received $CD4^+$ T-cell passive transfer. This phenomenon has been observed earlier (47) and immunohistological examination of infiltrates revealed the presence of $CD4^+$ T cells and macrophages but not that of $CD8^+$ T cells in antibody-treated rats (8).

DISCUSSION

Adoptive transfer of a $CD4^+$ T-cell line specific for the 24-kDa BDV antigen into virus-infected and CY-immunosuppressed rats results in either death within a few days or BD-associated neurological symptoms. Transfer of the cell line into infected rats which had been treated with MAb directed against $CD8^+$ or all T cells did not result in disease. Passive transfer also revealed enhanced kinetics of antiviral antibody synthesis. In addition to the phenotypic characterization, PCR analysis of the T-cell line revealed a cytokine pattern indicative of T-helper cells. No cytotoxic activity was found on MHC class II-matched virus-infected and IFN-γ-treated target cells or on NK-sensitive YAC-1 cells.

We have previously shown that $CD8^+$ T cells are an integral component of the immunopathological reaction in BD (reviewed in reference 45). The role of $CD4^+$ T cells in BD has been demonstrated earlier in experiments with a $CD4^+$ T-cell line specific for the 38- to 39-kDa antigen (reviewed in reference 44).

Since $CD8^+$ T cells generally have never been successfully established as lines or clones from rats, in this report we have reviewed the importance of $CD4^+$ T cells in BD in view of our recent reports on the role of $CD8^+$ T cells. Therefore, a homogeneous 24-kDa virus-specific $CD4^+$ T-cell line was generated. This noncytotoxic T-cell line and derived clones (data not shown) exhibit a helper cytokine profile and enhance the immunoglobulin response; hence, they are functional helper T cells. The characteristics suggest the presence of an intermediate or unusual phenotype of T-helper cells (17, 31a, 34, 36).

The most interesting feature of P205 T cells is the impact on the cell-mediated immune reaction after transfer. Experiments presented in this communication revealed two different patterns of disease, dependent on the time of transfer after CY treatment. When transfer of T cells was carried out at late time points after the initial immunosuppression, i.e., 10 to 15 days after CY, recipients died within 7 to 9 days without neurological symptoms but with a drastic loss of body weight of up to 30% within 3 days. Macroscopical and histological examination revealed signs of a severe lymphoproliferative disease of the

TABLE 5. Adoptive transfer of P205 $CD4^+$ T-cell line into anti-T-cell MAb-treated, BDV-infected recipients[a]

MAb	Cells transferred	Encephalitis		Body wt change (%)
		No. of rats with disease/total	Mean score ± SEM	
OX-8	P205	0/4	0	+>30
OX-8	None	1/4	0.1 ± 0.25[b]	+>30
OX-8	Immune spleen	4/4	2.4 ± 0.3[c]	−15
OX-52	P205	0/4	0	+>30
OX-52	None	0/4	0	+>30
OX-52	Immune spleen	4/4	2.5 ± 0.4[d]	−18

[a] Recipient rats were subjected to adoptive transfer of 5×10^6 P205 T cells or 10^7 spleen cells from diseased BDV-infected donors. Recipients were treated with MAb OX-8 (directed against $CD8^+$ cells) or OX-52 (directed against all T cells) on days −1 and +1 relative to the day of infection. The cells were transferred on day 21 p.i.
[b] The individual scores were 0, 0, 0, and 0.5.
[c] The individual scores were 2.0, 2.5, 2.5, and 2.5.
[d] The individual scores were 2.0, 2.5, 2.5, and 3.0.

TABLE 4. Effect of early transfer of P205 $CD4^+$ T-cell line into BDV-infected recipients[a]

No. of cells transferred	No. of rats with symptoms/total	Day p.t.	Encephalitis		T cells present in:		Body wt change (%)
			No. of rats with encephalitis/total	Mean score ± SEM	Brain	Lungs, intestines	
10^6	6/6	14	6/6	1.5 ± 0.4[b]	CD4, CD8	None	−23
10^6	6/6	16	6/6	1.75 ± 0.3[c]	CD4, CD8	None	−30
10^6	6/6	19	6/6	2.0 ± 0.4[d]	CD4, CD8	None	−29

[a] Recipient rats were subjected to adoptive transfer of 10^6 P205 T cells on day 5 p.i., i.e., day 4 after CY immunosuppression, and killed at various time points thereafter.
[b] The individual scores were 1.0, 1.5, 1.5, 1.5, 1.5, and 2.0.
[c] The individual scores were 1.0, 1.5, 1.5, 2.0, 2.0, and 2.0.
[d] The individual scores were 1.5, 1.5, 2.0, 2.0, 2.5, and 2.5.

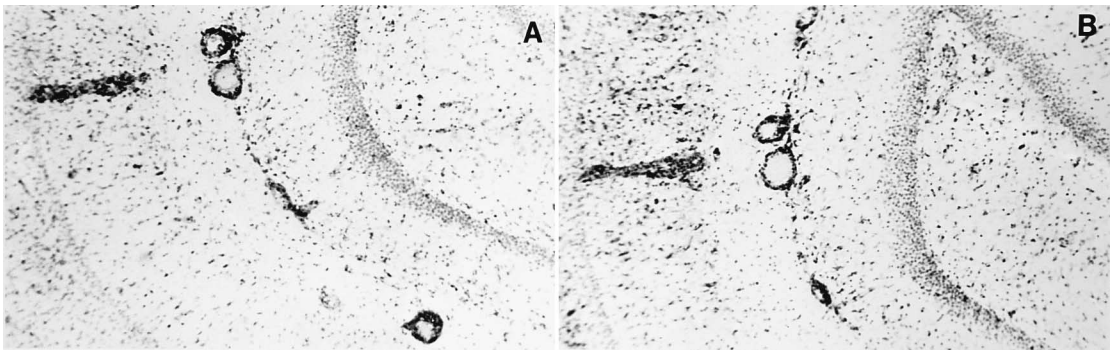

FIG. 5. Hippocampus region in the brain of a BDV-infected, immunosuppressed rat subjected to adoptive transfer of $CD4^+$ T-cell line P205 at day 4 after CY treatment. The presence of $CD4^+$ (A) and $CD8^+$ (B) cells as detected by MAb W3/25 and OX-8, respectively, is shown. Immunohistochemical staining was done by the ABC method. Magnification, ×80.

lungs, mesenteric lymph nodes, intestinal wall, and Peyer's patches. Since T cells in general and activated T-helper cells in particular are potent producers of cytokines and since transferred T cells, including virus-specific lymphocytes, preferentially migrate through the lungs and to the lymphoid tissue of the gut (4, 18, 19, 43, 55), it is likely that the observed inflammatory disease in peripheral organs is initiated by these cells. Interestingly, no BDV-specific antigen was found at this time in the guts or lungs of recipient rats. Therefore, we anticipate that transferred activated T-helper cells induce a fast and vehement but rather unspecific local immune response due to their cytokine synthesis and subsequent infiltration of other cell types into the reactive tissue. Migration of inflammatory cells into these areas is possible because the immune system has recovered, at the latest, by day 10 after CY treatment, when the adoptive transfer occurred. Cytofluorometric analyses of mesenteric lymph nodes revealed the presence of $CD4^+$ and $CD8^+$ cells 10 days after CY treatment; numbers and staining intensities of cells were comparable to those of cells from untreated rats, providing evidence of rather quick recovery of the immune system from the suppressive effects of CY (data not shown). In the brains of these rats, only slight inflammatory reactions were observed which, according to our experience, could probably not have caused rapid death without neurological symptoms. Therefore, we argue that the massive inflammation, especially in the intestines, caused the death of recipients before a BDV-specific immune response was generated in the brain.

In contrast, transfer of P205 $CD4^+$ T cells shortly after CY treatment, when the immunosuppressive effect does not allow sudden induction of a cellular host reaction because of the absence of lymphocytes, led to full-blown BD. In this case, in contrast to adoptive transfer late after CY immunosuppression, transferred T cells may be redistributed and finally migrate to the brain. In this respect, it is of importance that P205 T cells express the α4 integrin (VLA-4) which has been shown to be the crucial molecule that allows entry of activated T cells into the brain after binding to VCAM-1 on endothelial cells (5, 22, 57). In rats that received a passive transfer shortly after CY treatment, disease started around day 14 p.t. with a moderate loss of body weight and gradual development of neurological symptoms within the next few days resulting in ataxia and pareses. Immunohistochemical characterization of inflammatory cells in the brains of recipients of $CD4^+$ P205 T cells unanimously revealed the presence of about 20 to 30% $CD8^+$ T cells and more than 50% $CD4^+$ T cells with respect to the total number of lymphocytes.

In sharp contrast, recipients which had been thymectomized and treated with MAb against $CD8^+$ (OX-8) or all (OX-52) T cells prior to the transfer of P205 T cells did not show disease symptoms or notable encephalitic reactions (8, 47).

Recently, increasing evidence of the pathogenic importance of $CD8^+$ T cells in organ diseases has been presented. A wide body of evidence in favor of the activity of $CD8^+$ T cells in other virus infections of the brain has also been presented. In lymphocytic choriomeningitis virus, herpes simplex virus, West Nile virus, and Theiler's virus infections in mice (24, 30, 31, 33, 61); human immunodeficiency virus and human T-cell leukemia virus infections in humans; and Visna virus infections in sheep, $CD8^+$ cytotoxic T lymphocytes have been shown or are suspected to be a deleterious factor in the disease and/or to contribute to neurological disorders (16, 26, 27, 41, 49, 54, 56, 58).

Our own observations revealed that the presence of functional $CD8^+$ T cells in the brains of BDV-infected rats was paralleled by development of neurological disease, upregulation of MHC class I antigen in the brain, and MHC class I-restricted virus-specific cytotoxic activity in vitro (8, 35, 46, 47). Perivascular inflammations in BD encephalitis, in addition to macrophages, are predominantly composed of $CD4^+$ T cells of which the majority might be antigen-nonspecific, host-derived cells, as it has been shown in other models (1, 2, 13), since their presence, in the absence of $CD8^+$ T cells in parenchymal and perivascular infiltrations, has no pathological impact (8, 46, 47); furthermore, despite the high numbers of $CD4^+$ T cells in the brain (15, 38, 46), no considerable MHC class II-restricted killing, compared with high MHC class I-restricted lysis, was found in lymphocyte preparations isolated from the brains of BDV-infected rats (35). Finally, IFN-γ, which is secreted by cytotoxic T lymphocytes (34, 50) after they encounter their antigen in vivo, has also been detected in the brains of rats with BD (40). This cytokine activates macrophages and induces a delayed-type hypersensitivity reaction—in part from nonspecific host inflammatory cells—that finally results in tissue injury. In this context, it is of interest that both Th1 and Th2 cells have been shown to provide help to cytotoxic T lymphocytes and trigger production of IFN-γ by cytotoxic T lymphocytes (51). Since BDV is a noncytopathic virus that by itself does not cause detectable destruction in vitro or in vivo (20, 32), the cytotoxic T-cell response appears to be a major pathway to release of virus-specific antigen from infected cells, which might lead to promotion of the immune response, including induction of $CD4^+$ T cells. Our earlier and present observations on BD demonstrate that different types of $CD4^+$

T cells can be induced, represented by $CD4^+$ T-cell line NM1, which apparently has direct pathological effects (38, 39), and by $CD4^+$ T-cell line P205, which provides help. Therefore, these two different T-cell lines and their different in vivo effects upon adoptive transfer might reflect the broad spectrum of $CD4^+$ T cells found in vivo.

In conclusion, this work employing a T-helper cell line specific for a major BDV antigen provides additional evidence of the importance of virus-specific $CD8^+$ T cells in BDV-induced encephalitis that results in brain atrophy and chronic debility. As demonstrated also in other virus infections, virus-specific $CD4^+$ T cells apparently act as helpers and contribute to the immune reaction on the T- and B-cell level. Dependent on the virus studied, in vivo experiments have revealed a mandatory role for T help in the induction of a cytotoxic T-cell response in some viral infections (3, 6, 28, 29, 60). Our findings support the notion that actions of CD4 and CD8 subsets are required for antiviral delayed-type hypersensitivity responses. BD in rats appears to be a $CD4^+$ T-cell-dependent immunopathological disease in which $CD8^+$ T cells and/or $CD8^+$ T-cell-mediated cytodestructive mechanisms are operative.

ACKNOWLEDGMENTS

We thank Rudolf Rott for encouragement, support, and hospitality; Martin Sobbe for MAb treatment of rats; and Silke Gommel for outstanding technical assistance. Ian Lipkin and Heiner Niemann kindly provided recombinant virus proteins.

This work was supported by the Deutsche Forschungsgemeinschaft (DFG-Forschergruppe "Pathogenitätsmechanismen von Viren," Sti 71/2-1 and Bi-323/2-2) and the EC Network "Pathogenesis of subacute and chronic inflammatory diseases of the central nervous system." L. Stitz is a recipient of a Hermann and Lilly Schilling Professorship for Theoretical and Clinical Medicine.

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