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Precursors of Borna disease virus–specific T cells in secondary lymphatic tissue of experimentally infected rats

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Borna disease in rats represents an experimental model to study the immunopathogical role of T cells in central nervous system disease. Adoptive transfer experiments were performed to investigate homing properties of T cells that infiltrate the brains of infected animals. Lymphocytes isolated from the brains of diseased rats were labelled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) and transferred into immunosuppressed infected recipients. In recipient rats displaying neurological disease, labeled lymphocytes were demonstrated in the vicinity of brain cell lesions, suggesting that the neuronal destruction was dependent on the presence of transferred lymphocytes. Furthermore, the presence of virus-specific cytotoxic T cells was scrutinized in secondary lymphatic tissue and the functional activity of lymphocytes isolated from spleens, cervical lymph nodes, and mesenteric lymph nodes of infected animals was tested immediately after isolation and after *in vitro* restimulation. The data presented here indicate that precursors of Borna disease virus (BDV)-specific $CD8^+$ T cells are present and cytotoxic activity was demonstrated after *in vitro* cocultivation with infected cells in cervical lymph nodes and spleens but not in mesenteric lymphoid tissue. Adoptive transfer of *in vitro* restimulated T cells induced alterations in BDV-infected, immunosuppressed rats that resemble the well-defined clinical symptoms and neuropathology of Borna disease. This report provides for the first time formal evidence that virus-specific cytotoxic T cells are primed in the periphery after BDV infection, a disease that exclusively manifests itself in the central nervous system. *Journal of NeuroVirology* (2003) **9,** 325–335.

Keywords: Borna disease virus; cytotoxicity; encephalitis; immunopathology; T cells

Introduction

Experimental infection of Lewis rats with the highly neurotropic Borna disease virus (BDV) induces an immune-mediated neuropathological disease (Borna disease, BD) that is characterized by intense inflammation and massive degeneration of the brain (Narayan *et al*, 1983a; Hirano *et al*, 1983). $CD4^+$ and $CD8^+$ lymphocytes as well as macrophages and B lymphocytes are involved in the immunopathological reaction (Deschl *et al*, 1990). So far, pathogenetic pathways had been demonstrated by either abrogating T-cell responses by immunosuppressive agents, immunoregulating molecules, or specific antibodies directed against the CD4 or CD8 molecule or a Pan T-cell marker (Stitz *et al*, 1989, 1991, 1992; Planz *et al*, 1993). Major histocompatibility complex (MHC) class I–restricted $CD8^+$ T cells represent effector cells responsible for the acute-phase disease, as characterized by cellular degeneration in the brain, as well as the late-phase disease, as characterized by a distinct cortical brain atrophy (Bilzer and Stitz, 1994; Sobbe

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et al, 1997). In addition, T-cell lines were established that further defined the roles of virus-specific T cells in BD (Richt *et al*, 1989, 1990; Planz *et al*, 1995; Nöske *et al*, 1998). However, because $CD8^+$ T cells from rats have never been established as lines or clones *in vitro*, the use of cultured T cells so far was restricted to the use of $CD4^+$ T cells. By using these T-cell lines, some important features of the T cell–mediated immune response could be established, such as the induction of the disease-causing $CD8^+$ T-cell response after adoptive transfer of the $CD4^+$ cells, which alone did not exert any pathological reaction (Planz *et al*, 1995). Furthermore, $CD4^+$ T-cell lines were most helpful in defining the protective potential of T cells (Richt *et al*, 1994), a phenomenon that was later assigned to the action of $CD8^+$ T cells (Nöske *et al*, 1998). The important role of T cells was further verified when it was shown that the nucleoprotein p40 of BDV acts as a major target of the $CD8^+$ T cell–mediated immune response, and, finally, when the naturally processed MHC class I–associated peptide ASYAQMTTY was demonstrated as the target structure for the action of virus-specific $CD8^+$ T cells (Planz and Stitz, 1999; Planz *et al*, 2001). However, so far, only the presence but neither the sites of generation of cytotoxic T cells nor their homing properties to the brain have ever been investigated. In this paper, we have therefore investigated the homing of labeled and adoptively transferred lymphocytes isolated from the brain of BDV-diseased animals. Furthermore, in various peripheral lymphoid tissues, T-cell and precursor activities were tested by using cytotoxic T-cell assays, either directly after isolation of potential effector T cells or after secondary or tertiary restimulation, with virus-specific antigen in the presence of presenter cells. Here, we provide results indicating the peripheral priming of virus-specific T cells and the presence of precursor T cells for cytolytic activity in some but not in all secondary lymphoid tissue.

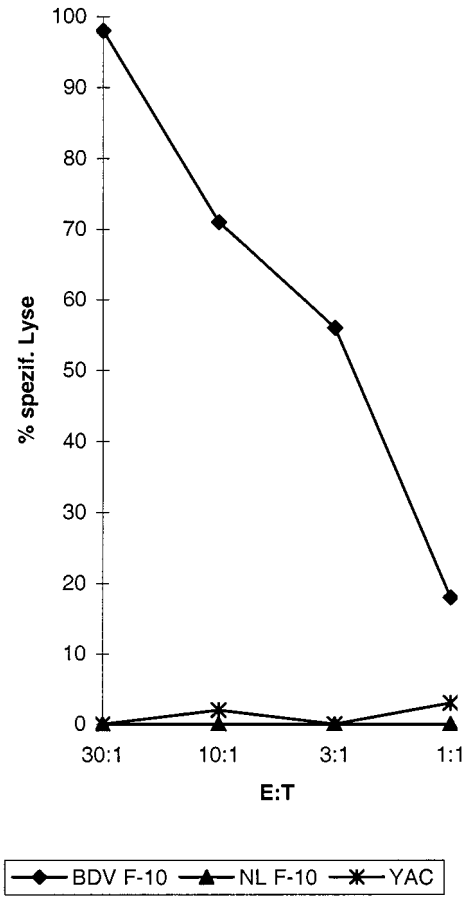


Figure 1 Percentage of specific lysis of BDV-infected (◆) and noninfected syngene target cells (▲) and YAC cells (✱) mediated by brain lymphocytes of diseased rats. Spontaneous release: BDV F-10: 22%; YAC: 16%; NL F-10: 21%.

Results

Homing of adoptively transferred brain lymphocytes to the brain in recipient rats

In earlier studies, we had shown that adoptive transfer of lymphocytes isolated from the brain of diseased rats into immunosuppressed infected syngeneic recipients causes disease and induces degenerative brain lesions (Sobbe *et al*, 1997). Because the recipients were immune compromised, it seemed obvious that the transferred lymphocytes migrated into the brain and were responsible for the resulting pathology. To confirm this hypothesis, lymphocytes were isolated from the brains of diseased rats at day 20 post infection (p.i.) were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CSFE) and adoptively transferred. Successful labeling was controlled by fluorescence-activated cell sorting (FACS) analysis (data not shown). The phenotypic characterisation of those cells by FACS analysis revealed more $CD4^+$ than $CD8^+$ T cells (ratio 1.4:1). This cell population exerted high lytic activity against BDV-infected syngeneic target cells (Figure 1). After intravenous (i.v.) adoptive transfer of these cells ($2 \circ 10^5$ lymphocytes/animal), recipients were sacrificed every third day and tissue of several organs was collected for immunohistochemical examination. After day 12 post transfer (p.t.), most of the recipients showed distinct clinical symptoms, such as loss of body weight, ruffeled fur, and a decline in general health condition. Those symptoms resembled the early stage of experimentally induced BD. The majority (3/4) of immunosuppressed BDV-infected controls without transfer did not display any signs of disease within the 18-day period of observation. Immunohistological examination of the brains of all recipients exhibiting BD-specific symptoms revealed well-known neuropathological changes such as degeneration of brain cells (data not shown).

To determine whether adoptively transferred T cells migrate into the brain of infected recipients exclusively, or whether they infiltrate other tissues as well, a higher amount of virus-specific T cells was transferred into three cyclophosphamide-treated recipient rats in a second experiment. Each recipient received $1 \circ 10^7$ CFSE-labeled T cells, which again had high cytotoxic capacity (data not shown). In this experiment, all recipients exhibited distinct signs of BD early after adoptive transfer (between days 4 and 9) and were sacrificed soon after onset of disease (between days 8 and 10 after transfer). Immunohistological examination revealed pathological changes and the presence of CFSE-labeled lymphocytes in the brains of all recipients. Labeled T cells were detected in the meninges, neocortical brain parenchyma (Figure 2**a**, **b**), as well as in the hippocampus. However, no labeled cells were detected in any of the other examined tissues (spleen, lymph nodes, eye, lung, liver, heart, kidney).

Induction of cytolytic activity after in vitro *restimulation of lymphocytes from secondary lymphoid organs*

In an earlier study, we demonstrated that lymphocytes isolated from spleen, lymph nodes, or the blood of infected animals did not exert BDV-specific cytolytic activity (Planz *et al*, 1993). However, lymphocytes isolated from cervical lymph nodes cause BD-specific symptoms after *in vitro* restimulation in the presence of specific antigen and adoptive transfer (Sobbe *et al*, 1997). In the present study, T cells were isolated from cervical lymph nodes of BDV-infected rats at day 9 (CLN9) or day 13 (CLN13) after infection and cultured in the presence of MHC class I–bearing, persistently BDV-infected, syngeneic fibroblasts as stimulator cells. After 8 days of culture, massive destruction of stimulator cells was seen. Lymphocytes were harvested at this time point of cocultivation and tested in an *in vitro* cytotoxicity assay. Furthermore, lymphocyte isolated from cervical lymph nodes of BDV-infected rats at day 20 p.i. (CLN20) were only cultured overnight in interleukin (IL)-2–containing medium and tested for cytotoxicity in the same assay. In this context it is noteworthy that lymphocytes obtained from the brain of infected rats between days 18 and 21 usually display high cytotoxic activity. Both populations of secondary *in vitro* stimulated T cells (CLN9 and CLN13) revealed high BDV-specific cytolytic activity, whereas short-term cultured cervical lymph node lymphocytes from day 20 (CLN20) did not exert lysis of infected cells (Figure 3).

To determine, whether lymphocytes isolated from other lymphoid organs reveal cytolytic activity after secondary *in vitro* stimulation, lymphocytes from the spleen (SPL) and from mesenterial lymph nodes (ML) isolated at day 14 and at day 21 p.i. were tested. After only 1 day of culturing, none of the lymphocyte populations isolated from lymphatic tissue of infected rats (data not shown) and none of the lymphocytes obtained from lymphoid tissue of uninfected rats exhibited virus-specific cytolytic activity (Table 1). In contrast, SPL and CLN from infected animals harvested at days 14 or 21 p.i. and restimulated for 8 days *in vitro* mediated killing of BDV-infected target cells, whereas uninfected targets and the natural killer (NK) target cell line YAC-1 were not lysed. ML did not exert cytotoxicity even after *in vitro* restimulation (Table 1). Cytometric characterization of cells tested 1 day after isolation revealed

Table 1 Cytotoxic activity of lymphocytes originally isolated from cervical lymph nodes (CLN), spleens (SL), or mesenterial lymph nodes (ML) after 8 day *in vitro* restimulation

Experiment	*Animals*	*Cells origin*	*CD4/CD8 ratio after isolation*	*CD4/CD8 ratio after cultivation of lymphocytes*	*CTL % specific lysis on target cells*			
					BDV F-10	*YAC*	*NL F-10*	*E:T*
I	NL	CLN	3.2:1	6.7:1	0/0/6/0	0/3/—/0	0/0/0/0	7:1
	NL	ML	3.1:1	5.6:1	0/3/31/0	0/2/—/0	0/0/0/0	38:1
	NL	SPL	2.9:1	5.4:1	0/0/0/0	0/3/—/0	0/0/0/0	27:1
	BDV d14	CLN	3.3:1	4.5:1	51/37/22/0	0/3/2/0	7/—/0/0	32:1
	BDV d14	ML	3.9:1	6.3:1	8/2/2/	0/0/1/0	0/—/0/0	41:1
	BDV d14	SPL	2.9:1	1:1	30/23/15/4	3/3/2/0	0/—/0/0	8:1
II	NL	CLN	6:1	12.6:1	0/0/0/0	n.d.	0/0/15/1	30:1
	NL	ML	4.2:1	8.7:1	0/0/0/0	0/0/0/0	0/0/0/0	126:1
	NL	SPL	4.1:1	7.1:1	0/0/0/0	n.d.	0/0/0/0	30:1
	BDV d14	CLN	3.8:1	4:1	82/28/8/0	n.d.	0/0/0/0	28:1
	BDV d14	ML	4.2:1	11:1	2/0/0/0	n.d.	0/0/0/0	52:1
	BDV d14	SPL	4.2:1	2.8:1	62/17/0/0	n.d.	0/0/0/0	37:1
	BDV d21	CLN	3.6:1	7.9:1	50/13/0/0	0/0/0/0	0/0/0/0	60:1
	BDV d21	ML	3.2:1	6.3:1	0/0/0/0	0/0/0/0	0/0/0/0	52:1
	BDV d21	SPL	3.3:1	3.7:1	28/8/0/0	0/0/0/0	0/0/0/0	45:1

Note. In two independently performed assays, the functional activity of lymphocytes isolated from BDV-infected animals at various times after infection or from noninfected rats (NL) was tested. The CD4/CD8 ratios after isolation versus after cultivation of lymphocytes reflect changes during the culture period. All restimulated cultures were suspended in equal volumes and tested in 1:3 dilutions in the cytotoxicity assay. The E:T ratios give the actual ratios of effector to targets in the assay as recalculated on the basis of the FACS data in restimulated cultures. n.d. = not determined.

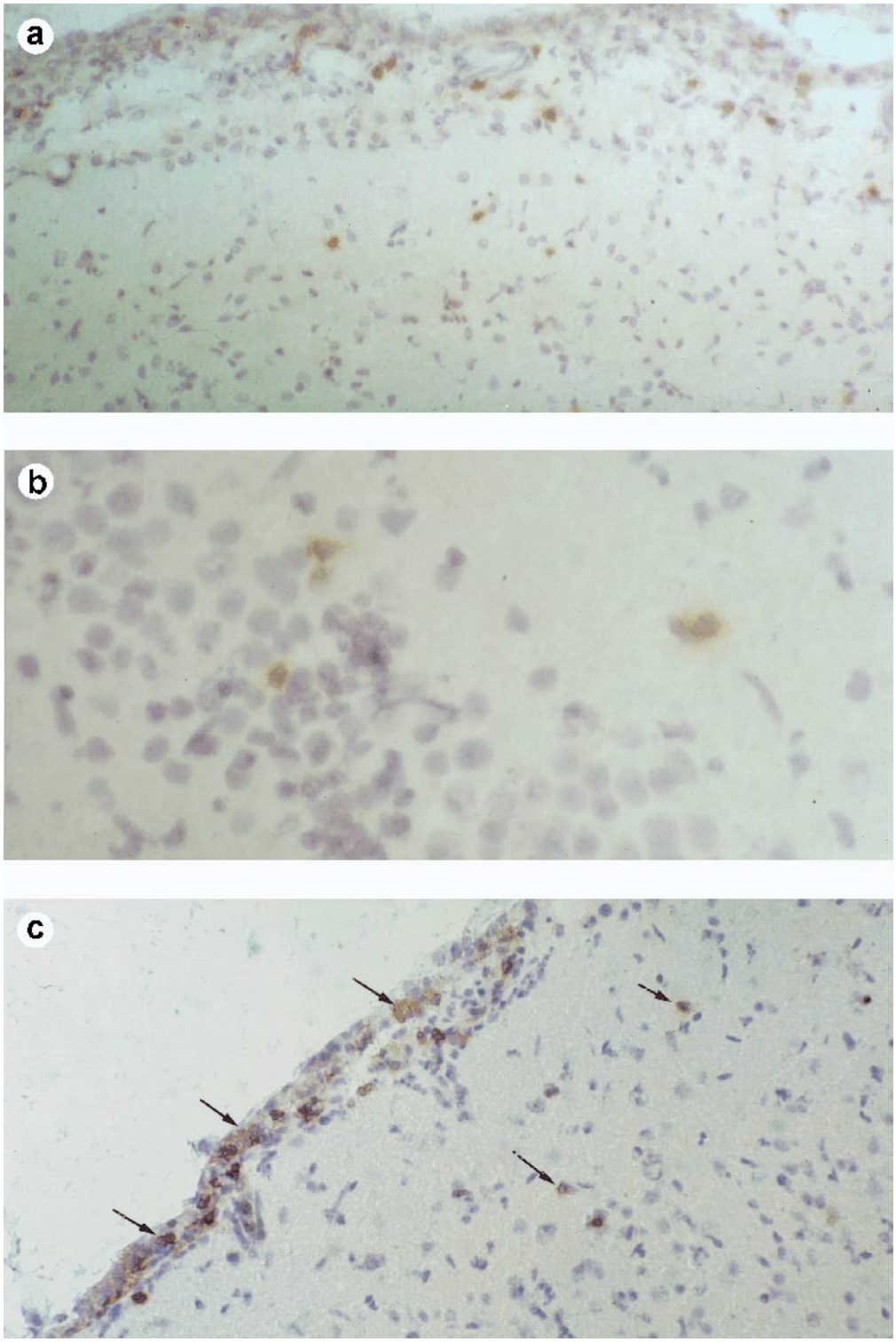


Figure 2 Detection of *in vitro* CSFE-labeled lymphocytes after adoptive transfer. (**a**) Brain lymphocytes from BDV-infected rats in the brain of immunocompromised BDV-infected recipient rats. (**b**) Labeled lymphocytes in the brain parenchyma. (**c**) Lymphocytes harvested from cervical lymph nodes and cultivated *in vitro*. Before transfer, lymphocytes were labeled with CSFE and visualization was performed by immunocytochemistry using an antibody against FITC in the peroxidase-diaminobenzidine reaction. ○ 120.

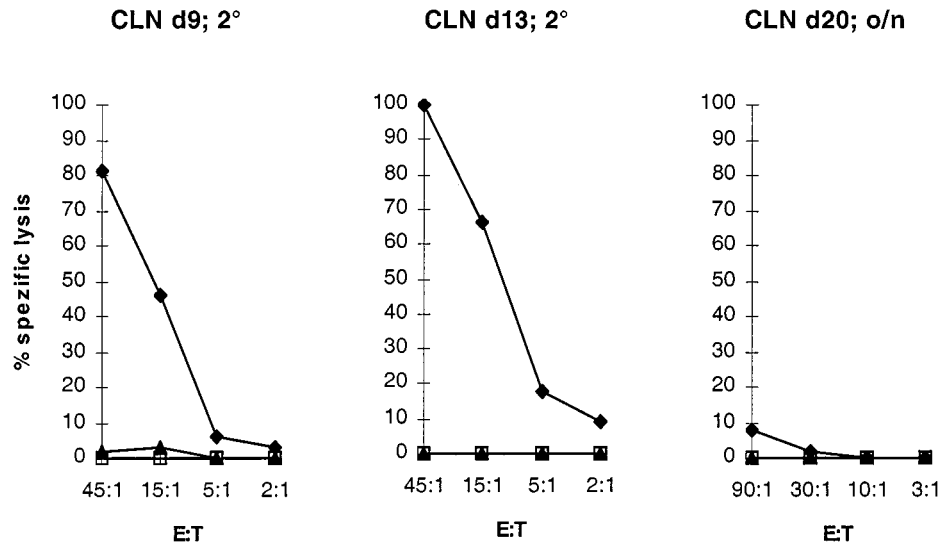


Figure 3 Lysis of infected (◆) and noninfected (▲) fibroblasts and YAC (□) cells mediated by in vitro restimulated lymphocytes originally isolated from the cervical lymph nodes (CLNs) of BDV-infected rats. The lymphocytes tested in assays A and B were restimulated for 8 days *in vitro*, whereas the T cells tested in assay C were isolated the day before and cultured overnight (o/n) in medium containing IL-2.

a 3:1 ratio of $CD4^+$ to $CD8^+$ T cells, whereas $CD4^+$ T cells usually had increased after *in vitro* restimulation (Table 2). In two experiments with restimulated cultures, it was shown that high virus-specific cytolytic activity was retained after a further 8-day *in vitro* restimulation (Table 2, tests A, D). However, in two other experiments, no lysis of infected targets was detected after the second *in vitro* restimulation (Table 2, tests B, C). Phenotypic characterization of the four different cultures showed that in cultures without cytolytic activity, the amount of $CD4^+$ cells had drastically increased, whereas the ratio of $CD4^+$ to $CD8^+$ cells was lower in cultures that still exhibited virus-specific cytotoxicity, i.e., in which more cytolytic $CD8^+$ T cells were present (Table 2). This result clearly demonstrates the difficulties in culturing rat lymphocytes and likewise makes it necessary to better define culturing conditions for rat T cells.

Table 2 Percentage of specific lysis of infected fibroblasts mediated by lymphocytes originally isolated from cervical lymph nodes at different times after isolation and the ration of $CD4^+/CD8^+$ T cells in the tested lymphocyte populations

		Cytotoxic activity Phenotype (CD4/CD8 ratio)		
Test	*T cells*	*Day 1 after isolation*	*After one restimulation*	*After two restimulations*
A	CLN 9	15/13/1/14 (4:1)	81/46/6/3 (9:1)	79/39/10/0 (8:1)
B	CLN 14	9/7/7/3 (3:1)	51/37/22/0 (3:1)	3/0/0/0 (36:1)
C	CLN 9	9/3/0/0 (4:1)	36/20/10/0 (9:1)	5/2/5/0 (85:1)
D	CLN 15	0/0/0/0 (5:1)	54/12/0/0 (5:1)	44/20/2/0 (7:1)

Note. The cytotoxic activity of the lymphocytes was tested shortly after isolation (day 1) and after one or two restimulations *in vitro*. The lymphocytes did not usually mediate lysis of noninfected fibroblasts or YAC cells.

To determine which T-cell population within restimulated lymphocyte cultures mediates lysis of infected targets, magnetic cell sorting for the enrichment of $CD8^+$ T cells was performed. Unseparated populations as well as CD8-enriched and CD8-depleted fractions were tested. Enrichment of $CD8^+$ lymphocytes resulted in enhanced lysis of infected target cells, whereas CD8-depleted populations did not exhibited any virus-specific cytotoxicity. Therefore, comparable to *ex vivo* isolated brain lymphocytes, lysis of infected target cells by *in vitro* restimulated lymphocytes appears to be mediated by $CD8^+$ T lymphocytes (Figure 4).

In vitro *restimulated lymphocytes cause Borna disease after adoptive transfer*

Adoptive transfer experiments were performed to test whether *in vitro* cultivated lymphocytes can induce BD. Lymphocytes isolated from cervical lymph nodes of rats at day 9 p.i. were restimulated *in vitro* and a cytotoxicity assay revealed the presence of virus-specific, cytolytic T cells in that culture (data not shown). The cells were labeled with CFSE and adoptively transferred into immunosuppressed-infected recipients (Figure 2**c**). Recipients had either been thymectomized and treated with anti–T cell antibody OX-52 or were treated with cyclophosphamid (Figure 5). Within 13 days after transfer, all recipients receiving restimulated lymphocytes showed moderate to severe clinical symptoms (9/9; Figure 5). Histological examination of brain tissue from diseased recipients revealed the presence of an inflammatory

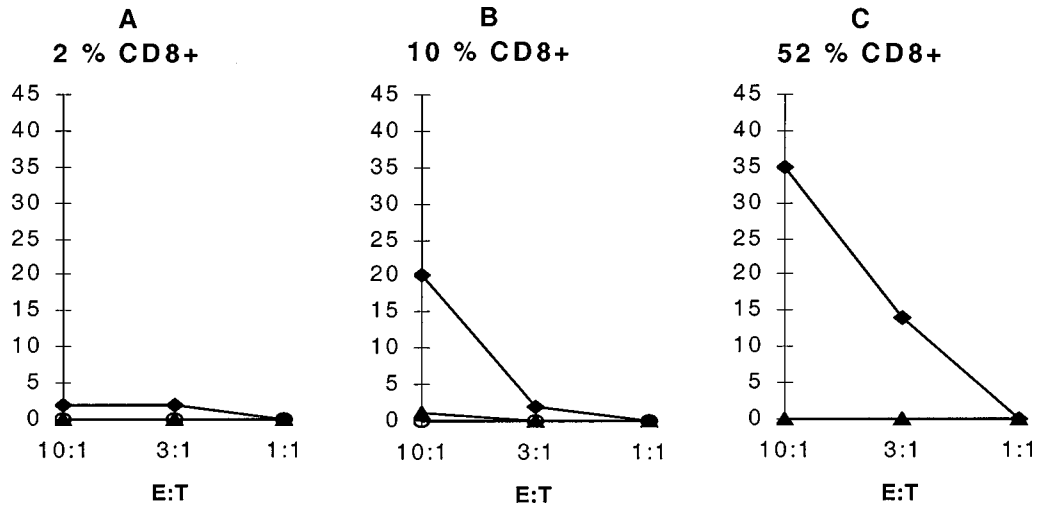


Figure 4 Lysis of infected (◆) and noninfected (▲) fibroblasts and YAC (□) cells mediated by *in vitro* restimulated lymphocytes originally isolated from cervical lymph nodes of infected rats and magnetically sorted to enrich the population of $CD8^+$ cells prior to the test. Comparison of the functional activity of CD8 depleted fraction (**A**), not separated culture (**B**), and CD8 enriched fraction (**C**). Spontaneous release after 10 h: BDV-Lew: 45%; YAC: 46%; NL-Lew: 42%.

response and neuronal degeneration that resembled changes during early stages of the disease in immunocompetent BDV-infected animals. None of the control rats with infection and immunosuppression but without transfer showed disease symptoms, neuropathology, or infiltrates during the 13-day observation period. Noninfected recipients of lymphocytes also did not show any symptoms or pathology (data not shown).

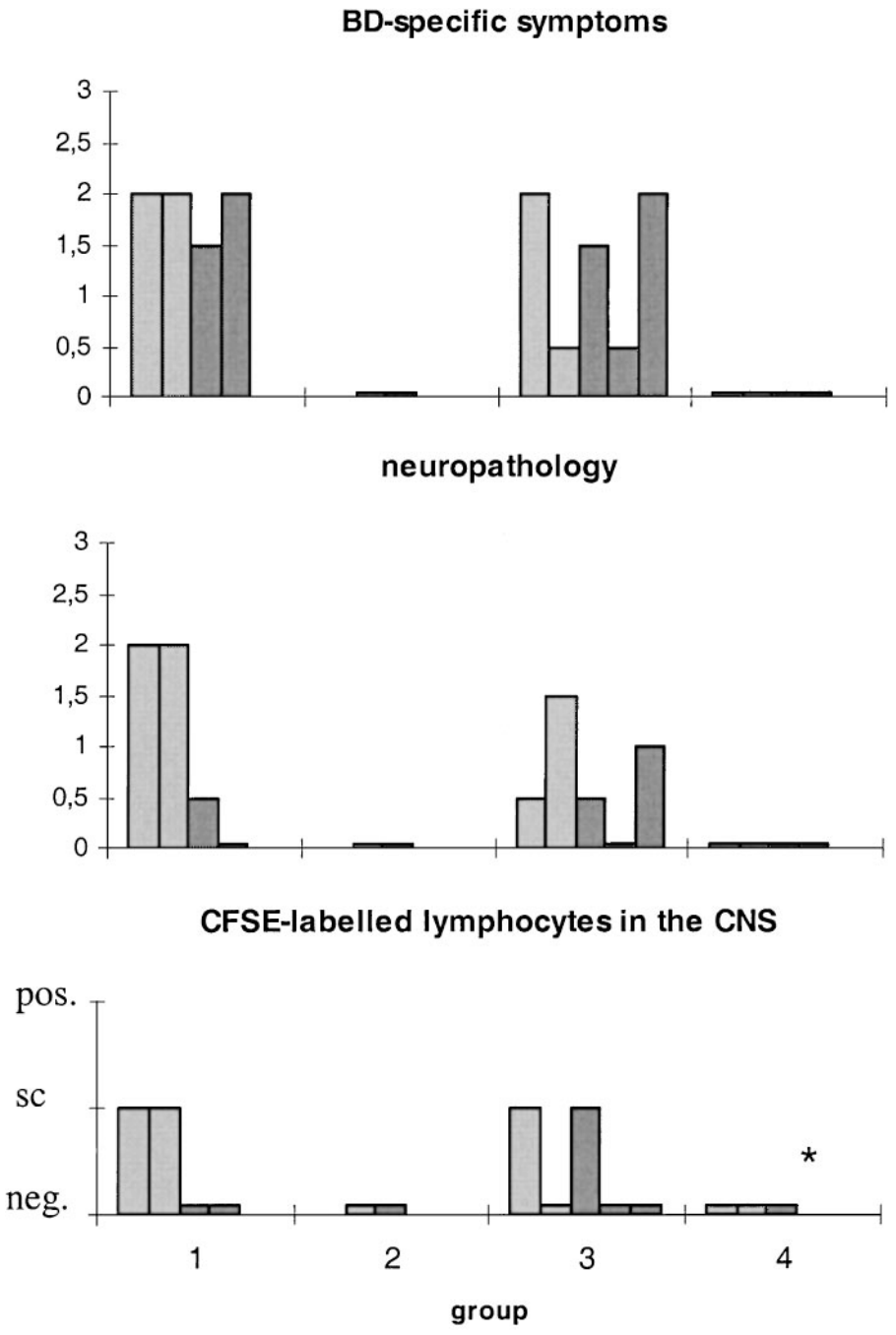
Discussion

BDV infection causes a neurological disease with intense inflammation based on an immunopathological reaction. It has been demonstrated in various studies that peripheral immunization with purified virus-specific antigen resulted in a humoral and cellular immune reaction and, as an experimental outcome, in the generation of $CD4^+$ T-cell lines capable of inducing disease (Richt *et al*, 1989, 1990; Planz *et al*, 1995; Nöske *et al*, 1998; Lewis *et al*, 1999; Hausmann *et al*, 1999).

Although BDV-specific $CD8^+$ T cells have been shown to represent the major effector T-cell population functional in the immunopathology of this brain disease, it was impossible so far to establish such lines or clones in rat models. Therefore, the present study aimed at determining the prerequisites for successful culturing of these pathogenetically relevant T cells. This included verifying peripheral T-cell priming after intracerebral BDV infection and determining the lymphoid organs that allow isolation of sufficient numbers of BDV-specific T cells for future work to culture BDV-specific $CD8^+$ cells. The use of lymphocytes from the periphery appeared to be the most appropriate source because lymphocytes isolated from the brain have been demonstrated not to be suitable for cultivation (Matsumoto *et al*, 1992). Although Rubin *et al* (1998) reported that anti-μ4 integrin antibodies suppressed the development of disease, suggesting that this was due to a reduced flux of BDV-specific lymphocytes from the blood to the brain, the site of priming of BDV-specific T cells and the presence of virus-specific T cells in lymphoid tissues had never been formally proven.

However, adoptive transfer of virus-specific lymphocytes from spleen (Narayan *et al*, 1983b) has been reported to cause BD and, furthermore, indirect evidence for peripheral priming of lymphocytes had been suggested after peripheral expression of BDV-specific antigen p40 by using vaccinia virus recombinants both in rats and mice (Lewis *et al*, 1999; Haussmann *et al*, 1999). It was also demonstrated that cells isolated from cervical lymph nodes or spleens and restimulated *in vitro* with specific antigen were capable of causing disease, though it was not shown whether the transferred cells themselves enter the brain and whether they exert cytolytic activity (Sobbe *et al*, 1997).

It is well established that intracerebral injection of agents immediately results in the passage of the injected material through the blood-brain barrier into circulation (Mims, 1960). Its subsequent passage through secondary lymphoid organs, such as spleen and lymph nodes, will induce a general immune response to eliminate the intruder from the organism by generation of a humoral and/or cellular response. To determine whether an efficient influx of lymphocytes obtained from the brains of diseased rats can be visualized, first adoptive transfers with CSFE-labeled cells were performed, which indeed



Treatment	AdTx	AdTx	Ox-52	Ox-52
Transfer	10^6	-	10^6	-
%CD8$^+$	10	-	10	-
n =	4	2	5	4

Figure 5 BD-specific symptoms, neuropathology, and presence of CFSE-labeled lymphocytes in the brains of BD-infected, immunosuppressed recipients of *in vitro* restimulated lymphocytes (groups 1 and 3, 1 ○ 10^6 lymphocytes, including 10% of CD8$^+$ cells/animal) and not transferred controls (groups 2 and 4). Animals of groups 1 and 2 had been adult thymectomized (AdTx) and T cell depleted by OX-52 administration, whereas rats of groups 3 and 4 had been immunosuppressed by single administration of cyclophosphamid prior to infection. The transferred lymphocytes were originally isolated from cervical lymph nodes of infected rats at day 9 post infection and restimulated for 8 days *in vitro* in the presence of infected syngene fibroblasts. Recipients and controls were sacrificed either on day 10 (*light grey bars*) or day 13 (*dark grey bars*) after transfer. Sc = single cells; * = not done.

revealed the presence of stained cells in the brain. Although variable numbers of CSFE-positive cells were found in the meninges and the brain parenchyma, we cannot answer the question whether the traced cells represent a quantitative parameter for the number of transferred cells or whether the labeled cells were progeny of some cells entering the brain and proliferating there in the presence of viral antigen on appropriate presenting cells. Whether lymphocytes are capable of proliferating in the brain was controversially discussed in different model systems (Matsumoto *et al*, 1992; Inaba *et al*, 1987; Sedgwick *et al*, 1991). The low number of labeled cells found here at least does not argue for an extensive proliferation at that site. However, at least the presence of CSFE-labeled cells provides evidence for rapid influx of specific activated T cells, which were transferred, because they could be demonstrated early after transfer and because only activated T cells are capable of crossing the blood-brain barrier.

Therefore, after intracerebral infection, we have tried to demonstrate the presence of virus-specific T cells in spleen and lymph nodes. The functional cross-over of T cells into the brain will be the crucial event in causing disease in this anatomically privileged site. Cells obtained at different time points after infection from spleen and cervical and mesenteric lymph nodes were cultured in the presence of virus-specific antigen for 8 to 21 days, in one experiment even for 30 days, and were found to exert cytotoxic activity and even showed an increase in cytotoxicity; however, with time, i.e., after two or more restimulations, the efficiency of cytotoxicity usually decreased. Experiments using magnetic cell separation and successive testing in cytotoxicity assays revealed that $CD8^+$ T cells are mediators of cytotoxicity also in restimulated peripheral lymphocyte populations. In contrast, without restimulation, peripheral lymphocytes had never been shown to be cytolytic (Planz *et al*, 1993). Our data show that only precursor cells of cytotoxic effectors are present in the periphery. This is strengthened by the finding that even cervical lymph node cells obtained at day 20 after infection do not show cytotoxic activity *in vitro*; in contrast, brain lymphocytes harvested at the same time point exert strong cytotoxicity.

Finally, the *in vivo* efficiency of cultured lymphocytes originally obtained from cervical lymph nodes was tested after adoptive transfer. Therefore, adult thymectomized recipients were infected intracerebrally and additionally immunosuppressed either by treatment with the antibody OX-52 or were treated with cyclophosphamid (Narayan *et al*, 1983a; Stitz *et al*, 1992). By transferring 10^6 cultured cervical lymph node cells after one restimulation cycle *in vitro*, with a calculated number of 10^5 $CD8^+$ T cells based on FACS analysis, all recipients displayed BD-specific clinical symptoms and showed neuropathology in addition to the presence of CSFE-labeled lymphocytes in the brain. Interestingly, labeled cells were exclusively found in the brain and meninges but never in peripheral organs, such as the lung, which usually hold back transferred cells, again arguing for a specific and rapid influx into the brain.

The fact that only few of the CSFE-labeled lymphocytes could be detected at one given time point within the perivascular inflammatory infiltrates and individually disseminated within the brain parenchyma may have several reasons: (i) the—above all—relatively small number of adoptively transferred lymphocytes; (ii) the partition coefficient between the periphery and the brain; (iii) a peak of infiltration directly after transfer, i.e., within 24 h; and (iv) a rapid turnover of the transferred activated cells. However, the results clearly show that cultured, restimulated, BD-specific lymphocytes are fully functional and cause disease even at low cell numbers. However, we have not yet determined culture conditions that would allow the propagation of isolated $CD8^+$ T cells for long-term cultures resulting in lines or clones.

In conclusion, we have demonstrated that lymphocytes isolated from the brain are effector cells *per se* but lymphocytes obtained from the spleen or the cervical lymph nodes need to be restimulated to exert cytotoxicity. Secondary restimulated effector cells from the latter lymphoid tissue are capable of inducing full-blown BD upon adoptive transfer. The clinical picture and the pathological damage caused by transferred restimulated peripheral lymphocytes exactly resemble those in virus-infected diseased rats and those in recipients of isolated brain lymphocytes. In peripheral lymphoid tissue, however, only precursors of cytotoxic effector cells appear to be present, which obviously only can cause disease if they cross into the brain where they get restimulated at the site of virus replication by locally presented viral antigen. The fact that cells labeled before transfer can be found in the brain and even more in the vicinity of degenerative lesions supports the role of locally restimulated T cells in the pathogenesis of BD, and also shows the importance of peripheral lymphoid tissue in priming and perpetuating an immunopathological reaction that manifests itself exclusively in the brain.

Materials and methods

Virus and experimental animals

The Tübingen laboratory strain of BDV was used throughout the study. Five- to six-week-old Lewis rats were infected by intracerebral injection into the left hemisphere with $5 \circ 10^3$ 50% tissue culture infective dose of BDV.

Clinical evaluation

All experimental animals were examined daily and weighed, and disease symptoms were scored on an arbitrary scale from 0 to 3, based on the general state of health (scores: 0.25 to 0.5, ruffled fur and hunchback; 1, slight incoordination and fearfulness; 2, distinct ataxia or slight paresis; 3, paresis or paralysis).

Body weight on the day of the adoptive transfer was defined as 100% and the percentage of weight change was calculated.

Isolation of T cells from the rat brain
Lymphocytes from the brain of BDV-infected rats were isolated by a method previously described by Irani and Griffin (1991) and modified for the BDV infection of rats (Planz *et al*, 1993). The animals were anesthetized with diethylether and perfused intensively with balanced salt solution (BSS). The brain was isolated and homogenized carefully through a stainless steel mesh and collected in BSS containing collagenase D (0.05%), trypsin inhibitor (TLCK [Na-P-tosyl-L-lysine chloromethyl ketone]) (0.1 mg/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 milliliter 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's medium (IMDM) with 2% fetal calf serum, and the cells were counted for further use in transfer or cytotoxicity assays.

Isolation of T cells from lymphatic tissue
Lymphocytes from spleens, cervical lymph nodes, and gut-associated lymph nodes were obtained after homogenizing lymphoid tissue carefully through a steel mesh. The cells were collected in BSS, washed twice, and 10 ml of the suspension was layered on top of 10 ml of a Lympholyte-Rat gradient and centrifuged at 1000 ○ *g* for 20 min. The interphase containing the lymphocytes was collected and the cells washed twice with BSS. The cells were counted and 1 ○ 10^6 cells were resuspended in 1 ml of IMDM, containing 5% rat sera and 5% supernatant of conavalin A–stimulated spleen cells.

Secondary in vitro *restimulation of lymphocytes*
In a secondary *in vitro* restimulation, 5 ○ 10^5 lymphocytes were cultured in the presence of 2 ○ 10^5 syngeneic fibroblasts (ratio 1:0.4). The fibroblasts were permanently BDV-infected, irradiated, and seeded into the wells of the 24-well plates the day before to allow them to adhere. On day 5, an additional 300 μl of fresh medium were added to the original 700 μl per well (IMDM, containing 5% rat sera and 5% supernatant of conavalin A–stimulated spleen cells). After 8 days, the lymphocytes were collected, washed once with BSS, and resuspended in either medium or buffer depending on the procedure following.

In vitro *cell-mediated cytotoxicity*
Aliquots of 10^7 virus-infected (BDV-F10 or BDV-Lew) and noninfected (F10 or Lew) histocompatible astrocytes (the astrocytic cell line cloned from a primary Lewis astrocyte culture, kindly provided by H. Wekerle, Munich, Germany) or fibroblasts were labeled with 0.2 mCi of ^{51}Cr at 37°C for 1 h and washed three times with medium. In some experiments, YAC cells were used as radioactive labeled target cells as well. The target cells were coincubated with *in vitro* stimulated or overnight cultivated T cells from BDV-infected or noninfected rats at various effector cell/target cell (E/T) ratios in a final volume of 200 ml/well. After 10 h, 50 ml 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.

Immunosuppression with cyclophosphamide or anti–T cell monoclonal antibody (mAb)
One day after intracerebral BDV infection, the animals received 160 mg of cyclosphosphamide per kilogram intraperitoneally. Alternatively, thymectomized rats were treated with 2 mg of purified mouse mAb directed against all T cells (OX-52) 1 day before and 1 day after infection (Stitz *et al*, 1992). After immunosuppression or antibody treatment, infected animals developed no signs of BD.

Adoptive transfer of lymphocytes
For adoptive transfer studies, *in vitro* stimulated lymphocytes isolated originally from cervical lymph nodes, or lymphocytes isolated from brains the day before, were injected intravenously into cyclophosphamide-treated recipients or into OX-52–treated, thymectomized recipients.

Labeling of T cells with CFSE
CFSE (Molecular Probes, Eugene, USA) was kept as a 0.5 mM stock in DMSO and stored at ○ 20°C. The T cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended at (1–2) ○ 10^7 cells/ml in ice-cold PBS. Cells were labeled by diluting the CFSE stock 1000-fold (final concentration 0.5 μM) and incubated at 37°C for 10 min. After labeling, fetal calf serum was added to 5% final concentration and the cells were immediately centrifuged at 200 ○ *g* for 10 min and washed twice with ice-cold PBS. Labeling of the cells was confirmed by cytofluorometry. For injection, the labeled cells were diluted at the indicated concentrations in BSS.

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

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 CFSE-labeled lymphocytes as described by Oehen *et al* (1997), by use of a polyclonal biotinylated goat antifluorescein antibody (Vector, Burlingame, CA, USA) followed by an avidin-biotin complex with peroxidase as marker enzyme (Vectastain; Vector). The reaction was visualized by 3,3-diaminobenzidine as substrate.

Cytofluorometric characterization of lymphocytes
Unlabeled and labeled lymphocytes were scanned on an FACscan flow cytometer (Becton & Dickinson). During the acquisition, the T-cell population was gated to exclude debris, and 10^6 cells were counted per sample. Cells were incubated with the following fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or Cy-Chrome–conjugated mAbs specific for leukocyte differentiation markers: OX-52 (T cells), OX-35 ($CD4^+$ T cells) and OX-8 ($CD8^+$ T cells) (all Beckton & Dickinson).

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