Antiviral CD8 T Cells Recognize Borna Disease Virus Antigen Transgenically Expressed in either Neurons or Astrocytes

Karen Baur,1†‡ Mathias Rauer,1† Kirsten Richter,1 Axel Pagenstecher,3 Jürgen Götz,2§ Jürgen Hausmann,1,* and Peter Staeheli1,**

Department of Virology, University of Freiburg, D-79104 Freiburg, Germany;1 Division of Psychiatry Research, University of Zürich, CH-8008 Zürich, Switzerland;2 and Department of Neuropathology, University of Marburg, D-35043 Marburg, Germany3

Received 17 November 2007/Accepted 24 December 2007

Borna disease virus (BDV) can persistently infect the central nervous system (CNS) of mice. The infection remains nonsymptomatic as long as antiviral CD8 T cells do not infiltrate the infected brain. BDV mainly infects neurons which reportedly carry few, if any, major histocompatibility complex class I molecules on the surface. Therefore, it remains unclear whether T cells can recognize replicating virus in these cells or whether cross-presentation of viral antigen by other cell types is important for immune recognition of BDV. To distinguish between these possibilities, we used two lines of transgenic mice that strongly express the N protein of BDV in either neurons (Neuro-N) or astrocytes (Astro-N). Since these animals are tolerant to the neo-self-antigen, we adoptively transferred T cells with specificity for BDV N. In nontransgenic mice persistently infected with BDV, the transferred cells accumulated in the brain parenchyma along with immune cells of host origin and efficiently induced neurological disease. Neurological disease was also observed if antiviral T cells were injected into the brains of Astro-N or Neuro-N but not nontransgenic control mice. Our results demonstrate that CD8 T cells can recognize foreign antigen on neurons and astrocytes even in the absence of infection or inflammation, indicating that these CNS cell types are playing an active role in immune recognition of viruses.

The Borna disease virus (BDV) model system can be used to study antigen presentation to MHC-I-restricted CD8 T cells in the CNS of rodents (2, 12). BDV is an enveloped virus with a single-stranded RNA genome of negative polarity (4, 7, 45) that can readily establish persistent infections of the CNS in a wide variety of vertebrate species (29, 41). BDV infects mainly neurons and rarely also astrocytes, Schwann cells, and ependymal cells (6, 10, 11, 39). Antiviral CD8 T cells play a central role in Borna disease. On the one hand, CD8 T cells are responsible for neurological disease which follows infection with BDV (2, 12, 35, 46). On the other hand, if present in sufficient amounts before BDV has massively spread through the CNS, CD8 T cells are able to restrict virus propagation very efficiently (14). The majority of CD8 T cells in the CNS of diseased mice recognize a single H-2k-restricted epitope (amino acid sequence 129TELEISSI136) of the BDV nucleoprotein (N) (44). TELEISSI-specific CD8 T cells use gamma interferon (IFN-γ) but not perforin to control the infection and to eliminate BDV from neurons (14, 16).

A key question in understanding the pathology of Borna disease and other viral diseases of the CNS is how CD8 T cells are able to recognize infected cells. It is conceivable that they detect viral epitopes presented by rare MHC-I molecules on the surface of infected neurons or astrocytes. Alternatively, they may detect viral protein fragments that are cross-presented on noninfected oligodendrocytes or microglia, which represent cell types that have been shown to express MHC-I molecules on the surface more efficiently than neurons (23, 37, 40). Since BDV is noncytolytic in vitro (20, 30, 43) as well as in vivo (11), it is difficult to reconcile the latter scenario.

We recently described transgenic mice that specifically and strongly express BDV N in either neurons (Neuro-N) or as-
trocytes (Astro-N) (39). Since these animals are tolerant to the neo-self-antigen, antiviral immune responses can be studied only after adoptive T-cell transfer. Here, we describe a reliable method for generating TELLEISSI-specific CD8 T-cell cultures which retain the ability to induce neurological disease if adoptively transferred into healthy nontransgenic recipients persistently infected with BDV. We report that the transfer of TELLEISSI-specific CD8 T cells readily induced neurological disease in transgenic mice expressing the cognate neo-self-antigen in either neurons or astrocytes, demonstrating that CD8 T cells are indeed able to recognize antigen on these CNS cell types.

MATERIALS AND METHODS

Mice. MRL/MpJ (MRL) and B10.BR mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding colonies were maintained in our local animal facility. Transgenic mice on the B10.BR genetic background expressing either Astro-N or Neuro-N have been described previously (39). B10.BR/Thy-1.1 mice were obtained by breeding congenic C57BL/6-Thy-1.1 mice (obtained from H. Pircher, University of Freiburg, Freiburg, Germany) with B10.BR/Thy-1.2 mice. All animal experiments were approved by the local authorities.

Viruses and animal infections. MRL and B10.BR mice were infected intracerebrally in the thalamic region of the left brain hemisphere by injecting 10 µl of a 10% brain homogenate containing 300 focus forming units of BDV into the thalamic region of the left brain hemisphere by injecting 107 PFU of recombinant vaccinia virus expressing BDV N, designated PPV-N. Construction and growth of D1701-VrV and parental strain D1701-VrV expressing β-galactosidase have been described previously (19). N-specific immunity was boosted 1 week postinfection by infecting animals intraperitoneally with 1 × 107 to 2 × 107 PFU of recombinant vaccinia virus expressing a FLAG-tagged version of BDV N (VV-N) (44). Two weeks after the booster immunization, mice were sacrificed, and spleens were removed for isolation and cultivation of N-specific CD8 T cells.

Generation of short-term CD8 T-cell cultures. Splenic lymphocytes from immunized mice were obtained by gently pressing the organ through a metal grid (60 mesh; Sigma). Responder splenocytes were seeded in Iscove’s modified Dulbecco’s (IMDM) supplemented with 10% fetal bovine serum, penicillin/streptomycin, and 1 mM β-mercaptoethanol (complete IMDM) into 24-well plates at 3 × 105 cells per well and mixed with 3 × 104 naïve splenocytes pulsed with 10-6 M TELLEISSI peptide (stimulator). TELLEISSI has previously been identified as the immunodominant CD8 T-cell epitope in H-2d mice (44). After 5 days of culture, recombinant murine interleukin-2 (IL-2) (Peprotech, New Jersey) was added to a final concentration of 25 U/ml. Two days later, all cells were harvested, pooled, and seeded again at 3 × 105 responders per well mixed with 3 × 104 TELLEISSI-pulsed stimulators. Cells were then incubated with one-third of conditioned medium from the first week of culture and two-thirds of fresh complete IMDM. Cultures were again supplemented with 25 U/ml of IL-2, incubated for another 7 days, and analyzed by flow cytometry using TELLEISSI-2K tetramer and anti-CD8α antibody. Only cultures containing at least 65% of TELLEISSI-specific CD8 T cells were used for adoptive transfer experiments. For in vitro activation of T cells specific for the glycoprotein epitope gp33 of lymphocytic choriomeningitis virus, spleen cells (5 × 105/ml) from gp33-specific T-cell receptor transgenic mice (27) were cultured in the presence of gp33 peptide (10-8 M) in 24-well plates for 3 days.

Peptides, MHC-I tetramer, and flow cytometry. Peptides were purchased from NeoSystem (Strasbourg, France) at a purity of ≥65%. TELLEISSI-2K tetramer complexes labeled with phycoerythrin were kindly provided by the National Cancer Institute (Frederick, MD). Tetramers (5 µg/ml for 107 to 108 lymphocytes in 50 µl) were used together with allophycocyanin-conjugated anti-CD8α antibodies (1 µg/ml; clone 53-6.7; BD Pharmingen). Incubation was for 30 min at room temperature. Analysis of stained cells was performed on a FACSort flow cytometer (BD).

In vitro cytotoxicity assay. Cytolytic activity of splenocytes was determined by standard 51Cr release assay with slight modifications, as described previously (44), using L929 (H-2b) cells as targets pulsed with 10-7 M TELLEISSI or the irrelevant H-2k-binding peptide FEANGNL derived from the hemagglutinin (HA) protein of influenza virus A/PR/8/34 (H1N1).

Adoptive transfer. Short-term cultures containing at least 65% of TELLEISSI-specific CD8 T cells were washed, pelleted, resuspended in serum-free IMDM, and filtered through a plastic mesh with a 100-µm pore size. Filtered cells were counted, pelleted, and resuspended in serum-free IMDM at the desired concentration. For intracerebral transfer, a maximal volume of 10 µl was injected into the left brain hemisphere of anesthetized adult mice using a Hamilton syringe. A maximum of 200 µl of cell suspension was injected into the tail vein for intravenous transfer.

Depletion and positive selection of lymphocyte subsets. CD4 or CD8 T-cell subsets were depleted from short-term cultures using anti-CD4- or anti-CD8-coated magnetic Dynabeads (Dynal Biotech, Hamburg, Germany). Depletion efficiency was controlled by flow cytometry of depleted cultures. Positive selection of CD8 T cells was performed using magnetic microbeads coated with anti-CD8 antibody and MS columns (Miltenyi, Bergisch Gladbach, Germany). Cultures were filtered through a 100-µm-pore-size plastic mesh before being applied to the MS columns. CD8 T-cell enrichment was controlled by flow cytometry.

Scoring of neurological disease and immune cell infiltration. Severity of neurological disease in BDV-infected mice was scored as follows: 0, no symptoms; 1, low degree of ataxia, increased anxiety; 2, clear ataxia, torticollis, unphysiological and uncontrolled movements of extremities when the animal was lifted by the tail, rough fur or hunched posture; 3, pronounced weight loss, severe ataxia, inward folding of hind limbs when the animal was lifted by the tail, torticollis, paraparesis, apathy, moribund. Infiltration of the brain with mononuclear cells was scored as follows: 0, no infiltrates; 1, up to two perivascular infiltrates per brain section with one or two layers of cells; some mononuclear cells in meninges close to five perivascular infiltrates per brain section with multilayer appearance and spread into parenchyma, intermediate meningitis; 3, six or more perivascular infiltrates per brain section with multiple layers of cells and strong infiltration of parenchyma at multiple sites, strong meningitis.

Histology and immunohistochemical analysis. Brain sections and immunohistochemistry were done as described previously (9). Viral load in CNS and transgenic expression of N were assessed by immunohistochemical staining of 8-µm paraffin-embedded brain sections with monoclonal antibody Bo18 directed against the viral nucleoprotein. Immune cell subsets in brains were analyzed by immunostaining of frozen mouse brain sections fixed with a 1:1 (vol/vol) mixture of methanol-acetone. Air-dried sections were incubated with anti-CD4, anti-CD8, anti-Thy-1.1, and anti-CD11b (Mac-1) antibodies. Immunostained sections were counterstained with Mayer’s hematoxylin.

RNA protection assay. Brains from diseased and healthy mice were removed at the same time point and snap-frozen in liquid nitrogen. RNA isolation using whole brains and RNA protection assays were performed as described previously (21). Briefly, 8 µg of total RNA was used for each sample and hybridized with a probe set containing probes for IL-1α, IL-1β, tumor necrosis factor alpha (TNF-α), TNF-β, IL-2, IL-3, IL-4, IL-5, IL-6, and IFN-γ. For the analysis of chemokine gene expression, 8 µg of the same RNA samples was hybridized with a probe set containing probes for CCL4, CCL5, CCL8, CCL11, CCL10, and XCL1. A probe for the RPL32-α gene served as an internal loading control. Biomax films (Kodak) were exposed for various periods of time and scanned using a ScanJet 4C (Hewlett Packard). NIH Image software, version 1.62, was used to quantify the autoradiographs.

RESULTS

TELLEISSI-specific short-term T-cell cultures. BDV-specific CD8 T cells were required for adoptive transfer experiments. Brains of severely diseased MRL mice do not represent a reliable source of such immune cells as they cannot usually be isolated in sufficient numbers. Therefore, CD8 T cells specific for peptide TELLEISSI, which represents the immunodominant BDV T-cell epitope of BDV in H-2b mice (44), were induced in B10.BR mice using a heterologous prime-boost immunization protocol that employs two different recombinant poxviruses expressing N protein of BDV N(14). The mice were first immunized with PPV-N and 1 week later with VV-N (Fig. 1A). Antiviral CD8 T cells were subsequently enriched by repeated stimulation of cultured splenocytes with peptide TELLEISSI. Splenocyte cultures from freshly immunized mice usually contained less than 10% TELLEISSI-specific CD8 T cells as deter-
mined by flow cytometric analyses using a tetrameric TELEISI/H-2Kk complex (denoted tetramer) (Fig. 1B, left panel). Cytolytic activity of such cultures was low (Fig. 1C, left panel). After 1 week of in vitro stimulation with peptide, more than 50% of the CD8 T cells present in the cultures were usually specific for TELEISSI (Fig. 1B, middle panel), and the cytolytic activity of such cultures was drastically increased (Fig. 1C, middle panel). After another round of stimulation with peptide, the percentage of TELEISSI-specific CD8 T cells increased further to usually more than 70% (Fig. 1B, right panel), concomitant with a decrease in the number of nonspecific immune cells (data not shown) and a decrease in background cytotoxic activity (Fig. 1C, right panel). Mainly because of the strong increase in T-cell specificity during the second week of culture, a protocol was adopted that included two rounds of in vitro stimulation of splenocytes from immunized mice with peptide TELEISSI at weekly intervals (Fig. 1A).

**TELEISSI-specific T cells induce disease in persistently infected mice.** Samples of short-term cultures containing various doses of TELEISSI-specific CD8 T cells were adoptively transferred into B10.BR mice persistently infected with BDV (designated B10-BDV). Such mice usually remain healthy in spite of large amounts of BDV antigen in the brain because their T cells mostly ignore the viral infection (15). All 22 B10-BDV
mice that received $10^5$ or more TELEISSI-specific CD8 T cells by intracerebral injection quickly developed severe neurological disease and had to be euthanized within 3 days after transfer (Fig. 2A). With doses of $5 \times 10^5$ and $1 \times 10^6$ cells, most animals developed severe neurological symptoms within 2 days after transfer, whereas disease induction was delayed for 1 day when $10^5$ cells were applied (Fig. 2A). Infected animals that received only $10^4$ specific T cells remained healthy during the whole observation period of 12 days (Fig. 2A). Symptoms of diseased animals included ruffled fur, hunchback, ataxia, and apathy, thus resembling the symptoms observed in MRL mice that get ill after infection with BDV (12). In contrast to B10-BDV mice, none of the eight uninfected B10.BR mice that received $10^6$ TELEISSI-specific CD8 T cells developed neurological disease (Fig. 2A, control).

To exclude possible artifacts resulting from intracerebral T-cell application, we tried to induce disease in B10-BDV mice by intravenous transfer of TELEISSI-specific CD8 T cells. This route of administration probably mimics the situation in virus-infected animals and may thus be considered more physiological. Greater numbers of CD8 T cells were used for the intravenous transfers because we assumed that only a minor fraction of the transferred cells would reach the brain (48). After intravenous transfer of $2 \times 10^7$ TELEISSI-specific CD8 T cells, neurological disorder was observed in all eight B10-BDV mice (Fig. 2B). Disease induction was delayed after intravenous application compared to intracerebral T-cell transfer and delay increased with decreasing numbers of transferred cells (Fig. 2B). When $1 \times 10^7$ TELEISSI-specific T cells were infused, 11 of 13 B10-BDV mice developed neurological symptoms. Transfer of $5 \times 10^6$ TELEISSI-specific T cells induced disease in only one of four B10-BDV mice (Fig. 2B). All 12 uninfected B10.BR mice (controls) that received the highest dose of TELEISSI-specific T cells remained healthy during the whole observation period of 14 days (Fig. 2B). We concluded from these results that cultured CD8 T cells with specificity for TELEISSI are able to induce neurological disease in persistently infected but not uninfected B10.BR mice after intracerebral and intravenous transfer. Progression to disease after adoptive transfer via the intravenous route was slower and required approximately 100 to 200 times more of the specific CD8 T cells than disease induction by intracerebral application.

To determine whether CD8 T cells alone are sufficient to induce disease in B10-BDV mice or whether they require help from CD4 lymphocytes, cultures with a cell concentration corresponding to $1 \times 10^6$ TELEISSI-specific CD8 T cells per dose were depleted for either CD8 or CD4 lymphocytes using suitable magnetic beads. Flow cytometric analyses demonstrated that approximately 3% of the CD8 T cells and less than 1% of the CD4 T cells remained in the depleted cultures (data not shown). All 11 B10-BDV mice that received CD4-depleted...
T-cell cultures developed disease within 4 days after intrace- 
rebral cell transfer. By contrast, seven of eight B10-BDV mice 
that received CD8-depleted cultures remained healthy during 
the whole observation period of 12 days (Fig. 2C), highlighting 
the critical role of CD8 T cells.

TELEISSI-specific T cells induce disease in transgenic mice 
expressing BDV N. BDV mainly infects neurons of mice, but 
infection of astrocytes does also occur (10, 39). We have pre-
viously generated transgenic mice expressing either Neuro-N 
or Astro-N (39). These animals are healthy, although viral 
antigen is abundantly present in the cognate cell types of the 
brain (39) (see Fig. 6G). Since neurons and astrocytes in non-
inflamed brains have few if any MHC-I molecules on the sur-
face (23), it was of great interest to determine whether TELE 
ISSI-specific T cells would recognize their cognate antigen in 
brains of transgenic mice.

Of the 17 Neuro-N mice that received $1 \times 10^6$ TELEISSI-
specific CD8 T cells, 14 developed disease within 10 days after 
intracerebral cell transfer (Fig. 3A). The onset of neurological 
symptoms was delayed compared to B10-BDV mice that re-
ceived similar numbers of TELEISSI-specific T cells by the same route of administration (Fig. 2A). Neuro-N mice further showed slower progression to ataxia. None of the seven nontransgenic control animals got ill under these experimental conditions. Neurological disease did not develop after intravenous transfer of approximately $10^7$ TELEISSI-specific CD8 T cells (Fig. 3B). Daily intravenous transfers of $2 \times 10^7$ TELEISSI-specific T cells over a period of 5 days similarly failed to induce disease in Neuro-N mice during the 14-day observation period (data not shown). Since direct injection of antiviral T cells into the brains of Neuro-N mice induced disease in most animals (Fig. 3A), antigen was obviously presented to CD8 T cells in such brains. Since uninfected brains of Neuro-N mice do not express chemokines (data not shown), the numbers of N-specific CD8 T cells entering the brains of Neuro-N mice after intravenous transfer might have been too low to reach local concentrations sufficient for initiating the disease process. We evaluated the possibility that danger signals triggered by physical damage following brain injection were critical for disease induction. However, brain wounding by mock injection combined with intravenous transfer of $1 \times 10^7$ to $3 \times 10^7$ TELEISSI-specific CD8 T cells did not change the picture. All seven Neuro-N mice used for this experiment remained healthy (data not shown).

Unlike Neuro-N mice, Astro-N transgenic mice express BDV N in astrocytes rather than neurons (39). All 41 Astro-N mice that received intracerebral injections of $10^5$ or more TELEISSI-specific CD8 T cells developed neurological disease within 2 to 5 days after transfer (Fig. 3C), whereas all 25 nontransgenic control mice remained healthy under these conditions. In contrast to Neuro-N mice, neurological disease progressed very rapidly, and animals usually had to be euthanized due to very severe disease within 1 to 2 days after onset of disease symptoms. To analyze the importance of antigen specificity of CD8 T-cell cultures, we injected $10^6$ short-term-cultured CD8 T cells specific for the irrelevant, H-2Kb-restricted gp33 epitope from the glycoprotein of lymphocytic choriomeningitis virus into Astro-N mice and nontransgenic B10.BR mice by the intrabrainal route. None of symptoms of neurological disease were observed in either group of mice upon transfer (Fig. 3F), indicating that antigen-specific responses were crucial for the disease process.

To determine whether the disease in Astro-N mice was caused mainly by CD8 T cells as in persistently infected B10-BDV mice, lymphocyte cultures were enriched for CD8-positive cells using suitable magnetic beads. Flow cytometric analyses demonstrated that the CD8 content in the enriched fractions was $\sim 90\%$ while it was reduced to $\sim 6\%$ in the remaining CD8-depleted cultures (data not shown). All seven Astro-N mice that received $10^6$ cells from the enriched fraction developed neurological disease after 2 days (Fig. 3E), whereas all seven Astro-N mice that received the remaining cells stayed healthy throughout the observation period of 18 days. Similarly, nontransgenic control mice that received $10^6$ cells from the enriched fraction remained healthy (Fig. 3E). These results indicated that CD8 T cells were the critical cell type for disease induction and that no other cell type appeared to have an important role in inducing neurological disease.

In contrast to the situation in Neuro-N mice, intravenous transfer of $1 \times 10^7$ to $3 \times 10^7$ TELEISSI-specific CD8 T cells into Astro-N mice induced disease in 14 of 15 recipients (Fig. 3D). Transfer by this route still induced disease in two of eight Astro-N recipients when only $5 \times 10^6$ CD8 T cells were applied. Thus, as in B10-BDV mice, intravenous application required at least 100 times higher numbers of adoptively transferred TELEISSI-specific CD8 T cells than intracerebral transfer to induce neurological disease. Interestingly, if neurological symptoms started to develop in Astro-N mice, they invariably turned very severe within 15 h of T-cell transfer. As expected if the observed effects were specific, none of the 10 nontransgenic control mice that received $1 \times 10^7$ to $3 \times 10^7$ TELEISSI-specific T cells became ill during the observation period of 16 days (Fig. 3D). We concluded from these results that astrocytes and neurons of uninfected mice can present peptides to CD8 T cells, although antigen presentation by neurons is seemingly less effective.

### Expression of inflammatory cytokines and chemokines in the CNS of transgenic mice after CD8 T-cell transfer

We performed RNase protection assays to analyze expression of inflammatory cytokines and chemokines as indicators of T-cell activity in brains of intracerebrally and intravenously transferred Astro-N mice. Brains of naïve Astro-N mice showed no detectable expression of a panel of inflammatory cytokines and chemokines (Fig. 4A and B; also data not shown). In contrast, increased expression of the inflammatory cytokines TNF-α, IFN-γ, IL-1α, and IL-1β was detectable in brains of diseased Astro-N mice intracerebrally transferred with $10^6$ TELEISSI-specific CD8 T cells. No cytokine-specific signals could be detected in brains of nontransgenic mice after identical T-cell transfer (Fig. 4A). Intracerebral transfer of $10^6$ TELEISSI-specific CD8 T cells also induced significant expression of CXCL10 (IP-10) and CCL2 (MCP-1), whereas expression of these chemokines was very low in brains of nontransgenic mice after T-cell transfer (Fig. 4B). Very weak CCL5 (RANTES) expression was detected only in brains of diseased Astro-N mice (Fig. 4B). After intravenous transfer, brains of Astro-N mice with severe neurological disease showed expression of CXCL10 but not CCL2 or any of the cytokines tested (Fig. 4C and data not shown). These results indicated that the transferred N-specific CD8 T cells exerted antigen-triggered effector functions in the CNS. The transferred CD8 T cells might have been the direct source of chemokine and cytokine expression detected in the CNS, or they might have induced expression of at least some of those molecules.

### Histological analysis of brains

Since intravenous transfer of TELEISSI-specific CD8 T cells into B10-BDV mice induced strong neurological disorder (Fig. 2B), the extent of brain mononuclear cell infiltration in such animals was of interest. In brains of healthy B10-BDV mice, immune cell infiltration is usually absent (15). This notion was confirmed in five of six animals used in our study (Fig. 5). The sixth exceptional animal presumably represented a missed case of mild disease in B10-BDV mice that we occasionally observe (unpublished results). In 17 of 18 B10-BDV mice that received intravenous infusions of TELEISSI-specific T cells, clear signs of immune cell infiltration were observed (Fig. 5). By hematoxylin and eosin staining, inflammatory cells were mainly detected around the major blood vessels (Fig. 6B). Since the TELEISSI-specific T cells used for these experiments originated from B10.BR mice that carry the Thy1.1 allele, specific antibodies could be used to
directly trace the fate of transferred T cells in Thy1.2-recipient mice (49). Thy1.1 antibody staining of brain sections from recipients with overt neurological disease revealed that a large number of transferred cells were present in the parenchyma of both cerebrum (data not shown) and cerebellum (Fig. 6C). The distribution pattern of transferred cells was similar to that of CD8 (Fig. 6D) but not CD4 (Fig. 6E) T cells, which tended to accumulate more frequently near the large blood vessels than the CD8 T cells. Thy1.1-positive cells were largely absent from the prominent inflammatory foci around the major blood vessels, indicating that the CD4 T cells observed in the brain tissue were mainly derived from the host. Cells with high levels of expression of MAC-1 (which presumably represent invading macrophages) were present mainly in the inflammatory foci around the large blood vessels (Fig. 6F). From these analyses it became clear that brains from diseased B10-BDV mice contained transferred T cells as well as immune cells of recipient origin, suggesting that the T-cell transfer terminated the state of immunological ignorance toward the persisting virus in B10-BDV mice.

Examination of brains from diseased Astro-N mice which received intravenous injections of TELEISSI-specific CD8 T cells revealed a strikingly different picture. By hematoxylin and eosin staining, no sign of immune cell infiltration was detected in any of the 17 Astro-N mice used for this experiment (Fig. 5 and 6H). Immunostaining indicated that the intravenously transferred Thy1.1-positive T cells failed to penetrate the brain parenchyma of Astro-N mice (Fig. 6I). These brains further contained almost no CD8 (Fig. 6J) and CD4 (Fig. 6K) T cells, and cells staining strongly with antibody MAC-1 were virtually absent (Fig. 6L). These results suggested fundamental differences in T-cell trafficking and neuropathogenesis between B10-BDV and Astro-N mice.

**DISCUSSION**

Since neurons and astrocytes reportedly carry few if any MHC-I molecules on the surface (8, 13, 25, 26, 32–34, 48), it remains largely unclear whether T cells are able to directly recognize viruses replicating in these cells or whether immune recognition of viruses in neurons and astrocytes is indirect and dependent on cross-presentation of viral antigen by other cell types of the CNS. To address this question, we took advantage of the BDV mouse model system for the following main reasons: (i) the infection of mice with BDV is limited to the central and peripheral nervous systems (12; also our unpublished results); (ii) BDV infections are noncytolytic, and the virus is spread mostly if not exclusively by cell-to-cell contacts (29); (iii) CD8 T cells mediate immunity to BDV (14, 16); (iv) the major CD8 T-cell epitope of BDV recognized by H-2b mice...
has been mapped (44); and (v) Neuro-N and Astro-N transgenic mice are available that strongly and selectively express the BDV N (which carries the major CD8 T-cell epitope) in neurons and astrocytes, respectively (39). We found that short-term cultures of epitope-enriched CD8 T cells recognized viral antigen in persistently infected animals as well as in noninfected mice that transgenically express BDV N in either neurons or astrocytes. To account for the finding that higher T-cell numbers were required to induce disease in Neuro-N mice and that the onset of disease in these mice was delayed compared to Astro-N mice, we assume that the efficacy of antigen presentation via MHC-I differs substantially between neurons and astrocytes. Altogether, our results indicate that although MHC-I molecules are difficult to detect by standard histochmical techniques on neurons and astrocytes in the noninflamed CNS, they are sufficiently abundant to mediate antigen presentation to activated antiviral T cells.

We note that a previous study with transgenic mice expressing HA of influenza A virus in astrocytes (5) yielded results that differ substantially from ours. Unlike the results observed in our study, intravenous transfer of HA-specific CD8 T cells failed to induce clinical signs of disease in HA-transgenic mice, although immune cell infiltration in the brain was clearly detected, with the peak incidence around day 5 after T-cell transfer. The observed differences are most likely due to the fact that our mice express the BDV N transgene fairly well, with intracellular antigen levels reaching roughly the levels of virus-infected cells (39). The concentration of transgenically expressed HA in astrocytes of HA-transgenic mice was presumably substantially lower, although quantitative data are lacking (5). It is thus conceivable that MHC-I-mediated presentation to CD8 T cells by astrocytes is only efficient if the antigen in question is present in high concentrations, as is typically the case in virus-infected cells.

FIG. 6. Immunohistochemical detection of intravenously transferred Thy1.1-positive CD8 T cells in mouse brains. Displayed are sagittal brain sections of diseased B10-BDV mice (A to F) and Astro-N mice (G to L) that received intravenous injections of approximately $10^7$ TELEISSI-specific, Thy1.1-positive CD8 T cells. Animals were killed when severe neurological symptoms were observed. Staining with monoclonal antibody Bo18 revealed preferential neuronal expression of BDV N in persistently infected B10-BDV mice (A) and astrocytic expression of transgene-encoded BDV N in Astro-N mice (G). Mononuclear cell infiltration was assessed after staining with hematoxylin and eosin (H&E). Insets show higher magnifications of areas around large blood vessels. Specific detection of transferred cells was performed using a Thy1.1-specific antibody. The presence of CD8 T cells, CD4 T cells, and macrophages and activated microglia (MAC-1) was monitored with specific monoclonal antibodies.
We noted that the intravenous transfer of TELEISSI-specific T cells into Astro-N mice induced neurological disease more quickly than intracerebral T-cell injection (compare Fig. 3C and D). This result was unexpected because the opposite behavior was observed if persistently infected mice were used as recipients. If the transferred T cells recognized targets in the brain, the kinetics of disease development should be enhanced rather than delayed after intracerebral application. We presently cannot satisfactorily explain this result. We previously showed that transgene expression is most prominent in the brain of our Astro-N mice and also clearly detectable in lung and intestinal tract (39). Therefore, the intravenously transferred T cells may have homed not only to the brain but also to other organs, and disease might thus have resulted from the action of antiviral T cells at these other sites. Since the clinical signs of disease in Astro-N mice that received antigen-specific T cells by either the intravenous or intracerebral routes appeared to indicate neurological disorder, this scenario appears unlikely. However, we cannot exclude the remote possibility that cytokines produced after recognition of antigen at peripheral sites have entered the brain and induced neurological symptoms. Both scenarios are compatible with our histological analyses, which failed to reveal overt brain infiltration in intravenously transferred Astro-N mice. Since strong illness developed in less than 20 h after intravenous transfer of T cells into Astro-N mice, it is probably not surprising that the transferred T cells were not detected in the brain parenchyma (Fig. 6G to L). In persistently infected mice where it was easy to identify the transferred cells (Fig. 6A to F), brain analysis was performed at 4 to 6 days after T-cell transfer because disease developed much more slowly. The finding of antigen-dependent induction of CXCL10 expression in Astro-N brains by intravenously applied N-specific CD8 T cells provides a clear indication that the antigen-specific CD8 T cells exerted activity in the CNS. Whether CXCL10 is directly involved in the pathogenic process or whether its expression is not relevant in disease induction and merely reflects CD8 T-cell activity that caused neurologic disease by other mechanisms remains open at present. The disease-inducing effector functions of BDV-specific CD8 T cells in BDV-induced neurological disease are still unknown. It became clear that BDV-induced neurological disease was dependent on CD8 T cells but not on perforin, IFN-γ, Fas, inducible NO synthase, or CXCR3, the CXCL10 receptor, as single factors (12, 17, 18). We believe that our findings with Astro-N mice could be explained by assuming that intravenously transferred TELEISSI-specific T cells preferentially attacked a subset of transgene-expressing astrocytes which were in direct contact with endothelial cells. An immune attack on these astrocytes might disturb the integrity of the blood-brain barrier, which is expected to result in severe neurologic disease. This kind of immune attack might not become histologically evident because interactions of N-specific CD8 T cells with N-expressing astrocytes might not be confined to certain brain areas but, rather, might be diffuse in nature. Of note, a similar lack of histological evidence of an immune attack by adoptively transferred CD8 T cells despite overt neurologic disease has been described in a model of lymphocytic choriomeningitis (1).

In the RIP-GP transgenic mouse model of autoimmune diabetes, it was shown that the inflammatory milieu strongly influences the susceptibility to organ-specific, T-cell-mediated immunopathology (28). The inflammatory environment differed in brains of BDV-infected and transgenic Astro-N or Neuro-N mice. Whereas the brains of the transgenic mice were immunologically silent (Fig. 4; also unpublished data), significant amounts of several chemokines including CXCL10 and CCL5 are produced in brains of BDV-infected mice independent of mononuclear cell infiltration (43). Moreover, studies in the immunologically tolerant newborn BDV-infected rat indicated that various cytokines like IL-6, IL-1α, IL-1β, and TNF-α were also induced as part of an intrinsic response of the CNS to BDV (38, 42). Thus, in BDV-infected brains, T cells are likely directed to areas of virus-induced chemokine production and further modulated by the local cytokine milieu, providing an explanation for the higher numbers of infiltrating immune cells in B10-BDV mice than in Astro-N mice after adoptive transfer of N-specific CD8 T cells.

ACKNOWLEDGMENTS

We thank Rosita Frank and Ginette Bortolussi for excellent technical assistance, the National Institute of Allergy and Infectious Diseases Tetramer Facility (Atlanta, GA) and the NIH AIDS Research and Reference Reagent Program for producing H-2K<sup>k</sup>-TELEISSI tetramer, and Hanspeter Pircher for providing access to gp33-specific CD8 T cells.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 620).

REFERENCES
