Central Nervous System Pathology Caused by Autoreactive CD8⁺ T-Cell Clones following Virus Infection

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Theiler's murine encephalomyelitis virus (TMEV) causes a demyelinating disease in infected mice which has similarities to multiple sclerosis. Spleen cells from TMEV-infected SJL/J mice stimulated with antigen-presenting cells infected with TMEV resulted in a population of autoreactive CD8⁺ cytotoxic T cells that kill uninfected syngeneic cells. We established CD8⁺ T cell clones that could kill both TMEV-infected and uninfected syngeneic targets, although infected target cells were killed more efficiently. The CD8⁺ T-cell clones produced gamma interferon when incubated with either infected or uninfected syngeneic target cells. Intracerebral injection of the clones into naïve mice induced degeneration, not only in the brain, but also in the spinal cord. This suggests that CD8⁺ Tc1 cells could play a pathogenic role in central nervous system inflammation.

Multiple sclerosis (MS) is the major cause of demyelinating disease of the central nervous system (CNS) in humans. Although the etiology of MS is unknown, virus infection and CNS organ-specific autoimmune responses are likely to play important roles in the pathogenesis. Similar to other organ-specific autoimmune diseases, major histocompatibility complex (MHC) class II-restricted CD4⁺ Th1-type responses have been extensively investigated in both MS and its experimental animal models (31). For example, experimental allergic encephalomyelitis can be induced by adoptive transfer of myelin-specific MHC class II-restricted CD4⁺ T cells into naïve animals. However, recent experimental evidence indicates that MHC class I-restricted CD8⁺ T cells are also involved in the pathogenesis of MS and its animal models (15, 17, 18, 24, 35). For example, CD8⁺ T cells have been shown to cause axonal injury, since CD8⁺ cytotoxic T lymphocytes (CTLs) can transect neurites in vitro (27).

Theiler's murine encephalomyelitis virus (TMEV) belongs to the family *Picornaviridae* and persistently infects glial cells and macrophages in the CNS after infection of susceptible mice, initiating an inflammatory demyelinating disease similar to MS (reviewed in reference 36). Although the precise mechanism(s) of demyelination in TMEV infection is not yet defined, demyelination can result from either direct viral infection of the myelin-forming cells, the oligodendrocytes, or an immune-mediated mechanism, or both. Immune-mediated mechanisms include TMEV antibody responses that cross-react with the myelin components, such as galactocerebroside (molecular mimicry) (10, 47) and delayed-type hypersensitivity responses to TMEV antigens with subsequent epitope (determinant) spreading to myelin antigens by CD4⁺ Th1 cells (reviewed in reference 43). CD8⁺ T cells have also been suggested to play an effector role in TMEV infection, since (i)

CD8⁺ T cells infiltrate the demyelinating lesions, (ii) in vivo administration of anti-CD8 antibody diminishes demyelination, and (iii) MHC class I molecules are upregulated in the CNS in TMEV-infected mice (reviewed in reference 8).

Previously, we demonstrated the induction of autoreactive CD8⁺ CTLs in SJL/J mice infected with the Daniels (DA) strain of TMEV (37). Spleen cells from TMEV-infected mice were stimulated with antigen-presenting cells (APCs) presenting TMEV antigens (TMEV APCs). The stimulated T cells were then used as effector cells in ⁵¹Cr release assays. These effector cells differed from conventional CTLs, since these T cells were able to kill both TMEV-infected and uninfected syngeneic cell lines but did not kill allogeneic cell lines. Since the TMEV-induced autoreactive cells killed neither the natural killer (NK)-sensitive cell line, YAC-1, nor NK-resistant target cell lines, P815 and EL4, they differed from NK cells or lymphokine-activated killer (LAK) cells induced by interleukin 2 (IL-2). Although LAK cells were cytotoxic for syngeneic target cells, LAK cells killed NK-sensitive, as well as NK-resistant, cell lines, both of which are allogeneic cell lines. The autoreactive killing required direct cell-to-cell contact and was mediated by a Fas-FasL pathway, not the perforin pathway. The phenotype of the killer cells was CD3⁺ CD4[−] CD8⁺. Injection of the autoreactive cells into the cerebral hemispheres of naïve mice caused meningitis and perivascular cuffing, not only in the brain parenchyma, but also in the spinal cord distant from the injection site. In the CNS, no viral-antigen-positive cells were detected by immunohistochemistry. This suggests that TMEV infection induced autoreactive cells that could play an effector role in TMEV CNS pathology.

From such autoreactive spleen cell cultures, we established CD8⁺ T-cell clones that were able to kill both TMEV-infected and uninfected syngeneic targets, although infected target cells were killed more efficiently. The CD8⁺ T-cell clones produced gamma interferon (IFN-γ) when incubated with susceptible target cells. Intracerebral injection of the clones into naïve mice caused inflammatory degenerative changes, not only in

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the brain, but also in the spinal cord. This suggests that CD8⁺ Tc1 cells play a pathogenetic role in the CNS.

MATERIALS AND METHODS

Establishment and phenotype of TMEV-induced CD8⁺ T-cell clones. We induced TMEV-specific autoreactive cells as described previously (37). Briefly, 3 weeks after infection with the DA strain of TMEV (6), spleen cells were isolated from female SJL/J mice (*H-2^b*; Jackson Laboratory, Bar Harbor, ME) and stimulated in vitro with TMEV APCs in RPMI 1640 medium supplemented with 10% fetal bovine serum for 6 days. TMEV APCs were isolated from NH₄Cl-treated spleen cells infected with DA virus at a multiplicity of infection (MOI) of 1 and irradiated with 2,000 rad using a ¹³⁷Cs irradiator. The TMEV-induced autoreactive cells were stimulated with TMEV APCs weekly. The cultured cells were treated with CD4 antibody (GK1.5; American Type Culture Collection [ATCC], Manassas, VA) (7) and LOW-TOX-M rabbit complement (Cedarlane, Hornby, Ontario, Canada) to deplete CD4⁺ T cells (11). CD8⁺ T cells were then enriched using a StemSep enrichment cocktail for murine CD8⁺ T cells (StemCell Technologies, Vancouver, British Columbia, Canada) according to the protocol supplied by the manufacturer. The enriched CD8⁺ T cells were cloned at 0.5 or 1 cell/well in flat-bottom 96-well plates containing 10⁶ stimulator TMEV APCs per well. The responder T cells were then restimulated with TMEV APCs weekly. IL-2 was added twice a week to a final concentration of 20 units/ml. One month later, vigorously growing clones were re-cloned at 1 cell/well into 96-well plates. The cells were transferred to 24-well and 6-well plates containing TMEV APCs and IL-2. Every 7 days, live cells were isolated using Histopaque-1083 (Sigma Diagnostics, Inc., St. Louis, MO), and 10⁶ T-cell clones were stimulated with 3 × 10⁷ TMEV APCs and 120 units of IL-2 in 6 ml of RPMI 1640 medium.

The T-cell clones were analyzed by FACScan and CELL Quest Software (Becton Dickinson Immunocytometry Systems, San Jose, CA), using monoclonal antibodies against CD3 (CT-CD3; Caltag, Burlingame, CA), CD4 (GK1.5; BD Biosciences, San Diego, CA), CD8a (CT-CD8a; Caltag), B220/CD45R (RA3-6B2; Caltag), and NK1.1 (PK136; BD Biosciences and Cedarlane).

Lymphoproliferative assay. A 200-μl volume of 10⁴ T-cell clones in RPMI 1640 medium supplemented with 1% glutamine, 1% antibiotics, 50 μM 2-mercaptoethanol, and 10% fetal bovine serum was added to each well of 96-well plates containing 2 × 10⁵ uninfected syngeneic APCs, TMEV APCs, or no cells. After the cells were cultured for 4 days, each well was pulsed with 1 μCi of [methyl-³H]thymidine, and the cells were then cultured for another 24 h. The cultures were harvested onto filters using a multiwell cell harvester, and ³H uptake was determined using standard liquid scintillation techniques (40). All cultures were performed in triplicate.

⁵¹Cr release assay. The syngeneic murine fibroblast cell line PSJLSV (PSJL) (*H-2^b*), was used as target cells in CTL assays. PSJL cells were kindly provided by Barbara Knowles (Jackson Laboratory) (22). PSJL cells express MHC class I, but not class II, antigens by flow cytometry (37). In some experiments, cells were infected with DA virus at an MOI of 5 for 18 h or were pulsed at an MOI of 10 with DA virus during the ⁵¹Cr loading of the target cells. Target cells were labeled with Na₂⁵¹CrO₄ (New Life Science Products, Inc., Boston, MA) for 1 hour. The target cells (10⁴) were placed in 96-well round-bottom plates. Effector cells were used at a concentration of 10⁶/100 μl, and threefold serial dilutions were made to provide effector-to-target cell (E/T) ratios of 100:1, 33:1, 11:1, 3:1, and 1:1. T-cell clones (effector) and target cells were incubated together for 5 hours, and the ⁵¹Cr released from the cells was measured using a Model 20/20 Iso-Data gamma counter (Iso-Data, Inc., Palatine, IL). The percentage of target cell lysis was calculated by the following equation: percent lysis = [(experimental-release cpm – spontaneous-release cpm)/(maximal-release cpm – spontaneous-release cpm)] × 100 (37, 41). Antibody-blocking studies to determine the MHC restriction of the killing were carried out at an E/T ratio of 1:1 in the presence of MHC class I antibody (34-1-2s; ATCC) (20, 29) or MHC class II antibody (Y3P; ATCC) (19). TMEV-induced CD8⁺ T-cell clones were incubated with uninfected PSJL cells in the presence of MHC class I or II antibodies.

Enzyme-linked immunosorbent assay. We measured IFN-γ and IL-4 production using an enzyme-linked immunosorbent assay, OptEIA Set (BD Biosciences), as previously described (41). T-cell clones were cultured at 2 × 10⁶ cells/ml in six-well plates in the presence or absence of concanavalin A (ConA) (5 μg/ml). Culture supernatants were harvested 48 h after stimulation. We also harvested supernatant fluid from T-cell clones stimulated with TMEV APCs and IL-2 after 1 week.

Adoptive transfer of T-cell clones. We adoptively transferred 2 × 10⁶ T-cell clones, 8a-1A and 8b-1C, in 30 μl of phosphate-buffered saline (PBS) into the right cerebral hemispheres of the naïve mice. As cell controls, we used activated spleen cells. Spleen cells were isolated from naïve SJL/J mice and treated with an

NH₄Cl (red blood cell-lysing) solution. The cells were cultured at 2 × 10⁶/ml with 1,000 U of recombinant mouse IL-2 (BD Bioscience) per ml and used as LAK cells (25, 37). One week after culture, a portion of LAK cells were treated twice with CD4 antibody (GK1.5) and complement to deplete CD4⁺ cells and used as CD8⁺ enriched LAK cells. We also activated spleen cells using ConA. Spleen mononuclear cells were isolated using Histopaque, treated twice with CD4 antibody and complement, and cultured with 5 μg/ml of ConA for 3 days and used as ConA-activated CD8⁺ T cells. Groups of five mice were injected intracerebrally with total LAK cells, CD8⁺ enriched LAK cells, or activated CD8⁺ enriched T cells. Mice were observed for 1 week and sacrificed, and CNS tissues were collected for histological analyses. As a negative control, we used normal spleen cells or PBS alone. Clinical signs of mice were evaluated by an impairment of the righting reflex (30, 42). When the proximal end of the mouse's tail is grasped and twisted first to the right and then to the left, a healthy mouse resists being turned over (score of 0). If the mouse is flipped onto its back but immediately rights itself on one side or both sides, it is given a score of 1 or 1.5, respectively. If it rights itself in 1 to 5 seconds, the score is 2. If righting takes more than 5 seconds, the score is 3. The mice were killed on day 7 posttransfer and perfused with 4% paraformaldehyde in PBS. CNS tissues were embedded in paraffin, and 4-μm-thick tissue sections were stained with Luxol fast blue for myelin visualization. Using immunohistochemistry, we identified oligodendrocytes and TMEV antigens using a carbonic anhydrase II (CAII) antibody (Binding Site, San Diego, CA) (39) and DA virus hyperimmune rabbit serum (38, 41), respectively. To demonstrate DNA fragmentation as a marker for apoptosis, we used terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL), using the FragEL DNA fragmentation detection kit (Oncogene, La Jolla, CA).

RESULTS AND DISCUSSION

Establishment of TMEV-induced CD8⁺ T-cell clones. To further characterize the TMEV-induced autoreactive cells, we established TMEV-induced CD8⁺ T-cell clones from TMEV-induced autoreactive spleen cell cultures stimulated with TMEV APCs. Initial attempts to establish CD8⁺ T-cell clones from the cultures by limiting dilution (28) were unsuccessful, since most of the T-cell clones were CD4⁺ CD8[−] (data not shown). To overcome this, we first depleted CD4⁺ T cells using CD4 antibody and then enriched for CD8⁺ T cells using a StemSep enrichment cocktail for murine CD8⁺ T cells (StemCell Technologies) before limiting-dilution isolation. To maintain cytotoxicity, supplementation with IL-2 was necessary, except during the first week of in vitro stimulation (21). Three clones, 8a-1A, 8a-1C, and 8b-1C, were selected for further characterization. These T-cell clones were analyzed by flow cytometry. The surface phenotype of the T-cell clones was CD3⁺ CD4[−] CD8⁺ B220^{dim} NK1.1^{dim} (Fig. 1).

TMEV-induced CD8⁺ T-cell clones require TMEV APCs for proliferation. Coculture of T cells with syngeneic cells has been reported to induce significant proliferation (syngeneic mixed-lymphocyte reaction [SMLR]) (2). The SMLR is considered to be driven by the T-cell response to self-antigens presented by MHC molecules on APCs. Aranami et al. (2) showed that the predominant dividing cells in the SMLR were CD8⁺ T cells. Previously, we demonstrated that spleen cells isolated from TMEV-infected mice proliferated when stimulated not only with TMEV APCs, but also with uninfected syngeneic spleen cells (syngeneic APCs; autoprolieration) (40). Conversely, the generation of TMEV-induced autoreactive cells requires in vitro stimulation using TMEV APCs, but not uninfected syngeneic APCs (37). We tested whether TMEV-induced CD8⁺ T-cell clones could proliferate when stimulated with TMEV APCs or uninfected APCs. As shown in Fig. 2, all the CD8⁺ T-cell clones proliferated when stimulated with TMEV APCs, but not when cultured in the presence of uninfected syngeneic

FIG. 1. The surface phenotype of TMEV-induced T-cell clones was CD3⁺ CD4⁻ CD8⁺ B220^{dim} NK1.1^{dim} by flow cytometry. All three T-cell clones, 8a-1A, 8a-1C, and 8b-1C, expressed similar cell surface markers. Data are shown for the T-cell clone used for the disease transfer experiment. FITC, fluorescein isothiocyanate.

APCs. Thus, T-cell clones required TMEV APC stimulation for efficient proliferation, while they responded weakly, if at all, to uninfected syngeneic APCs.

TMEV-induced CD8⁺ T-cell clones kill TMEV-infected and uninfected syngeneic targets. We used syngeneic PSJL cells as target cells in CTL assays. Interestingly, all three TMEV-induced CD8⁺ T-cell clones, 8a-1A, 8a-1C, and 8b-1C, killed not only TMEV-infected target cells, but also uninfected target cells, although the former were killed more efficiently (Fig. 3a and b). Thus, the clones displayed TMEV-specific, as well as syngeneic (autoreactive), cytotoxicity. To determine whether the syngeneic killing by TMEV-induced CD8⁺ T-cell clones was MHC restricted, we incubated the T-cell clones with uninfected PSJL cells at an E/T ratio of 1:1 in the presence of MHC class I or II antibody. Incubation with MHC class I antibody inhibited the killing of uninfected PSJL cells, while MHC class II antibody had no significant effect on killing (Fig. 3c).

TMEV-induced CD8⁺ T-cell clones produce IFN- γ , but not IL-4. We determined whether the T-cell clones produced Th1 (Tc1)- or Th2 (Tc2)-type cytokines by measuring IFN- γ and IL-4. In the culture supernatant fluid of T-cell clones stimulated with TMEV APCs and IL-2 for 1 week, IFN- γ was detected (T-cell clone 8a-1A, 1,000 pg/ml; T-cell clone 8b-1C, 930 pg/ml), but IL-4 was undetectable. After ConA stimulation, T-cell clones produced large amounts of IFN- γ (T-cell clone 8a-1A, 1,040 ng/ml; T-cell clone 8b-1C, 1,000 ng/ml), while little or no IL-4 was detected. Using the enzyme-linked immunospot assay (Murine IFN- γ Eli-spot; Diaclone Research, Besançon Cedex, France), we confirmed that the T-cell clones produced IFN- γ after incubation with TMEV-infected or uninfected syngeneic PSJL cells (data not shown).

FIG. 2. All three TMEV-induced CD8⁺ T-cell clones, 8a-1A, 8a-1C, and 8b-1C, proliferated in response to TMEV-infected APCs (*, <0.05, and **, <0.01 compared with effector-alone group). The T-cell clones proliferated less well to uninfected syngeneic APCs. All cultures were performed in triplicate. The results are means plus SEM of three to six experiments.

Adoptive transfer of T-cell clones produces CNS degeneration. We investigated whether TMEV-induced T-cell clones could initiate CNS pathology. T-cell clones 8a-1A and 8b-1C were transferred into naïve mice. During the 1-week observation period, 25% of the mice showed a mildly impaired righting reflex (mean righting reflex score of all mice injected with T cell clones $\pm$ standard error of the mean [SEM], 0.9 ± 0.1). Histologically, T-cell clones induced CNS pathology, including meningitis, perivascular cuffing, and gliosis in the corpus callosum, caudoputamen, internal capsule, basal forebrain, cerebellar peduncle, cerebellum, midbrain, and brain stem (Fig. 4a). Five of eight mice developed large CNS degenerative lesions with loss of myelin. Lesions were located not only close to the injection site, such as the thalamus, fimbria hippocampi, and corpus callosum, but also some distance from the injection site, including the posterior funiculus of the spinal cord (Fig. 4d). No difference was seen in CNS pathology between mice injected with the two separate CD8⁺ T-cell clones. Control mice that received normal spleen cells or PBS had no lesions but only mild gliosis around the injection site (Fig. 4c).

We occasionally observed fragmented nuclei in degenerative lesions, suggesting apoptosis of some cells. As seen in Fig. 4b and e, some nuclei in the lesions were TUNEL⁺. Using an oligodendrocyte-specific marker, CAII, we tested whether (i) a lack of myelin staining was associated with apoptosis of oligodendrocytes and (ii) apoptotic nuclei were those of injected T-cell clones or those of CNS cells. Combined TUNEL-immunofluorescent staining for CAII demonstrated that some TUNEL⁺ nuclei colocalized with CAII, while other TUNEL⁺ cells were CAII⁻ (Fig. 4g to i), suggesting that the TUNEL⁺ CAII⁺ cells were oligodendrocytes. The TUNEL⁺ CAII⁻ cells could be injected T-cell clones, other inflammatory or neuronal cells, or oligodendrocytes that lost CAII staining due to

FIG. 3. TMEV-induced CD8⁺ T-cell clones 8a-1A (a) and 8b-1C (b) were used as effector cells in ⁵¹Cr release assays. TMEV-infected or uninfected syngeneic PSJL cells were used as target cells. Both T-cell clones killed not only TMEV-infected syngeneic target cells, but also uninfected syngeneic cells, although the infected cells were killed more efficiently than uninfected target cells. The results are representative of four independent experiments. (c) TMEV-induced CD8⁺ T-cell clones were incubated with uninfected PSJL at an E/T ratio of 1:1 in the presence of MHC class I or II antibody. MHC class I antibody inhibited the killing of PSJL cells (left), while MHC class II antibody showed no significant inhibition of killing (right). The results are the means plus SEM of three experiments.

degeneration. Around the lesions, the intensity of CAII staining was markedly decreased, suggesting oligodendrocyte loss. No viral-antigen-positive cells were detected in the lesions or in other parts of the CNS, as determined by immunohistochemistry using TMEV antibody (Fig. 4f).

Finally, we tested whether injection of highly activated cells that have LAK activity could also cause similar pathological changes, regardless of specificity. Spleen cells from naïve mice were activated by ConA (ConA-activated cells) or IL-2 (LAK cells) and transferred intracerebrally into naïve mice with or without CD8⁺ enrichment. During the 1-week observation period, we did not observe clinical signs in any of the mice injected with ConA-activated CD8⁺ cells, total LAK cells, or CD8⁺ enriched LAK cells. Histologically, we detected mild gliosis and myelin pallor only around the injection site in the brains of some mice. No mice developed lesions in the brain or spinal cord distant from the injection site (Fig. 4j to l). In addition, we did not see large degenerative lesions comparable to those seen in mice injected with TMEV-induced autoreactive cells. This is compatible with the observation that there is little direct evidence for CNS degeneration *in vivo* by non-CNS-specific immune cells (26), despite various reports of bystander killing initiated by CD4⁺ (33, 45) or CD8⁺ (1) T cells *in vitro*.

While the precise mechanism(s) of CNS degeneration by TMEV-induced CD8⁺ T-cell clones *in vivo* is not known, one possible mechanism is recognition by CD8⁺ T cells of self-antigen presented by MHC class I molecules on target cells (oligodendrocytes) in the CNS, possibly through molecular mimicry between TMEV antigens and host proteins (9). MHC class I molecules are expressed by most cells in general organs. However, in the CNS, neurons, oligodendrocytes, astrocytes, and microglia do not express MHC class I molecules under normal conditions, while IFN- γ has been shown to induce or upregulate MHC molecules on glial cells, including oligodendrocytes (3). We demonstrated that TMEV-induced CD8⁺

T-cell clones can produce IFN- γ . Thus, upon intracerebral transfer of the CD8⁺ T-cell clones, T-cell clones themselves could secrete IFN- γ into the CNS parenchyma, leading to induction or upregulation of MHC class I molecules on glial cells, which enable T-cell clones to recognize self-antigens presented by MHC class I molecules on target glial cells. In addition, CNS trauma, which is caused by intracerebral injection in our current experiments, is known to induce MHC class I and II molecules into the CNS (13). This could also aid in the T-cell clone recognition of MHC molecules on target cells. We are currently investigating which self-antigen is recognized by the TMEV-induced CD8⁺ T-cell clones. For this purpose, we have generated cDNA libraries from TMEV-infected and uninfected CNS of SJL/J mice and have transfected portions of the library into uninfected astrocytes, which are not recognized by the TMEV-induced autoreactive CD8⁺ T cells. We are also investigating what TMEV epitope is recognized by TMEV-induced CD8⁺ T-cell clones. We are transfecting or infecting target cells with plasmid DNA or with vaccinia virus encoding capsid and noncapsid proteins of TMEV (34). Once we determine the TMEV epitope and self-antigen epitope that are recognized by the T-cell clones, we will test the extent of molecular mimicry between the epitopes.

Alternatively, the CNS degeneration could be induced in a bystander mechanism. Once the CD8⁺ T cells are activated by some other host cells presenting self-antigen or by a nonspecific stimulus, such as cytokines or superantigen, CD8⁺ T cells could kill neighboring oligodendrocytes that express death receptor molecules, such as Fas. We have previously demonstrated that the Fas-FasL pathway likely mediates TMEV-induced autoreactive cytotoxicity *in vitro* (37). Here, IFN- γ would aid in the activation of Tc1 cells or upregulation of MHC class I molecules in neighboring cells. In addition, IFN- γ itself could be the effector molecule for the CNS degeneration, since (i) IFN- γ induces apoptosis or necrosis in oligodendrocytes *in vitro* (3, 44), (ii) hypomyelination or primary demy-

FIG. 4. CNS degeneration caused by intracerebral adoptive transfer of TMEV-induced T-cell clones. Mice were killed 1 week after cell transfer. Transfer of the T-cell clones caused vacuolar degeneration (arrowheads), myelin loss, and gliosis, not only in the brain (a) (internal capsule [IC]), but also in the spinal cord (d) (posterior funiculus) distant from the injection site. The myelin sheath was totally lost and replaced with gliosis in the caudoputamen (upper left area above the internal capsule [a]) and at the center of the posterior funiculus of the spinal cord [d]). (b and e) In the lesions, TUNEL⁺ nuclei were detected (arrows) (counterstain, methyl green). (c) Control mice did not develop degenerative changes in the white matter. Myelin sheaths were preserved in the caudoputamen and internal capsule. (f) TMEV antigen⁺ cells were not detected in consecutive sections of the CNS by immunohistochemistry using the avidin-biotin peroxidase complex technique (counterstain, hematoxylin). (g) Apoptotic nuclei were labeled by TUNEL (fluorescein isothiocyanate, green). (h) Oligodendrocytes were visualized using antibody against CAII (rhodamine, red). (i) The merged image demonstrated two dual-positive cells for TUNEL and CAII (arrows; orange), while TUNEL single-positive cells (double arrowheads) and CAII single-positive cells were also present. (j to l) Transfer of total LAK cells (j), CD8⁺ enriched LAK cells (k), or CD8⁺ enriched ConA-activated cells (l) did not cause degeneration in the brain or spinal cord. Myelin sheaths were preserved in the internal capsule and caudoputamen (l) (upper left area of the internal capsule) and in the spinal cord (j and k). (a, c, d, and j to l) Luxol fast blue stain. (b, e, and g) TUNEL. (f and h) Immunostaining for TMEV (f) and CAII (h). Magnification: a to c and l, $\times 75$; d to f, j, and k, $\times 150$; g to i, $\times 260$.

lination was observed in transgenic mice that expressed IFN- γ in oligodendrocytes (4, 16), (iii) neutralizing antibody to IFN- γ reduced the severity of CNS autoimmune disease induced by myelin basic protein (MBP)₇₉₋₈₇-specific CD8⁺ T cells (17),

and (iv) IFN- γ -producing CD8⁺ T cells could mediate demyelination in mice infected with the coronavirus mouse hepatitis virus JHM strain, another murine model for MS (5).

In our experiments, TMEV-induced CD8⁺ T-cell clones

were expanded in vitro and induced CNS degeneration when injected into the CNS. Similar effector CD8⁺ T cells in vivo could participate in or cause CNS demyelination during TMEV infection. Potentially, certain TMEV-specific CD8⁺ T cells could kill TMEV-infected targets, as well as uninfected targets, if they express self-antigens that mimic TMEV presented in the context of MHC class I. The CD8⁺ T cells could also kill target cells indirectly in a bystander manner when these cells are in close proximity to TMEV-infected cells or areas of inflammation.

We feel that these CD8⁺ T cells are the counterparts of previously described CD4⁺ T cells isolated from MS patients that can recognize viral and self-epitopes (12, 14, 23, 32, 46). Our CD8⁺ T cells can recognize viral and self-epitopes but can also transfer disease to naïve animals. To our knowledge, this is the first description of virus-induced CD8⁺ T cells that can transfer inflammatory demyelinating disease to naïve animals.

The present study demonstrates that virus-specific CD8⁺ Tc1 clones can induce CNS pathology. To further define the role of these cells in CNS inflammation, we have generated CD8⁺ T-cell hybridomas from our TMEV-induced CD8⁺ T-cell clones (M. Kobayashi-Warren, I. Tsunoda, L.-Q. Kuang, J. E. Libbey and R. S. Fujinami, Abstr. Keystone Symp. Central Nervous Syst. Inflammation: Mechanisms, Consequences and Therapeutic Strategies, Snowbird, Utah, abstr. 101, 2005). Characterization of TMEV-induced CD8⁺ Tc1 clones and hybridomas will help in understanding the role of CD8⁺ T cells in CNS demyelinating diseases, including MS.

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