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Cytotoxic T Cells Isolated from the Central Nervous Systems of Mice Infected with Theiler's Virus

MARK D. LINDSLEY,[1] ROGER THIEMANN,[1] AND MOSES RODRIGUEZ[1,2]*

Departments of Immunology[1] and Neurology,[2] Mayo Medical School, Rochester, Minnesota 55905

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Intracerebral inoculation of resistant mice (C57BL/10SNJ) with Theiler's murine encephalomyelitis virus (TMEV) results in acute encephalitis followed by subsequent clearance of virus from the central nervous system (CNS). In contrast, infection of susceptible mice (SJL/J) results in virus persistence and chronic immune-mediated demyelination. Both resistance and susceptibility to TMEV-induced disease appear to be immune mediated, since immunosuppression results in enhanced encephalitis in resistant mice but diminished demyelination in susceptible mice. The purpose of these experiments was to determine whether anti-TMEV cytotoxic T lymphocytes (CTLs) are generated during acute and chronic TMEV infection. Nonspecific lectin-dependent cellular cytotoxicity was used initially to detect the cytolytic potential of lymphocytes infiltrating the CNS irrespective of antigen specificity. Using TMEV-infected targets, *H-2*-restricted TMEV-specific CTLs of the CD8$^+$ phenotype were demonstrated in lymphocytes from the CNS of susceptible and resistant mice, arguing against the hypothesis that the ability to generate CD8$^+$ CTLs mediates resistance. In chronically infected SJL/J mice, TMEV-specific CTL activity was detected in the CNS as late as 226 days postinfection. These experiments demonstrate that virus-specific CTLs are present in the CNS during both acute and chronic TMEV infection. Anti-TMEV CTLs in the CNS of chronically infected SJL/J mice may play a role in demyelination through their ability to lyse TMEV-infected glial cells.

Experimental infection of mice with Daniel's strain of Theiler's murine encephalomyelitis virus (TMEV), a picornavirus, results in a biphasic disease of the central nervous system (CNS) (24). Three to seven days after intracerebral TMEV infection, mice develop acute encephalitis as a result of neuronal infection (9, 41, 47). If virus persists following infection, chronic demyelination develops in the spinal cord in association with virus antigen in astrocytes, oligodendrocytes, and macrophages (9, 41, 47). Whereas all mice experimentally infected with TMEV develop acute encephalitis, persistence of virus and CNS demyelination occur only in some mouse strains. Demyelination produced by TMEV is under the control of genes which encode major histocompatibility complex (MHC) class I products (8, 38, 40). C57BL/10 congeneic mice expressing the *s*, *q*, *r*, *v*, *f*, or *p* haplotype are unable to clear TMEV infection and are susceptible to demyelination, whereas mice expressing the *b*, *d*, or *k* haplotype clear virus infection and are resistant to demyelination.

Destruction of oligodendrocytes (myelin-producing cells) may result from direct lytic infection by virus (45, 46). However, demyelination is also enhanced by the presence of an active immune response. Treatment of SJL/J (*H-2*^{s}) mice during the demyelinating phase of disease with immunosuppressive regimens results in fewer and less extensive CNS demyelinating lesions (25, 26, 39, 43, 44). CD8$^+$ T lymphocytes appear to be necessary for demyelination, since their depletion reduces the number and extent of demyelinating lesions (44). Conversely, immunosuppression of TMEV-infected SJL/J mice during the stage of acute encephalitis is detrimental. Depletion of CD4$^+$ T lymphocytes results in the increased frequency of fatal encephalitis and neuronal necrosis (44). In C57BL/10 (*H-2*^{b}) mice, which are normally resistant to demyelination, sublethal doses of irradiation at the time of virus inoculation result in increased frequency of encephalitis, viral persistence, and small areas of demyelination (42). Therefore, the immune response is important both in clearing virus from resistant mice and in the induction of demyelination in susceptible mice. Resistance is inherited as a dominant trait in C57BL/10 congeneic mice (35), which indicates that the immune response plays an active role in limiting virus infection. As a corollary, susceptible strains of mice appear to lack this ability to clear persistent infection.

We have proposed that a class I-restricted cellular immune response is the critical determinant of resistance versus susceptibility to TMEV-induced demyelination. Isolation and characterization of CNS (brain and spinal cord)-infiltrating leukocytes (CNS-ILs) during acute infection has revealed differences in the number of CD8$^+$ lymphocytes in the CNS (22) between mice that are resistant and those that are susceptible to chronic demyelination. Seven days postinfection (p.i.), CNS-ILs from resistant mice contain a predominance of CD8$^+$ T lymphocytes. In contrast, few CD8$^+$ T cells have been isolated from the CNS of susceptible mice during early infection, but these cells increase in number throughout chronic infection. On the basis of these findings, we proposed that resistance is due to the presence of class I-restricted CD8$^+$ cytotoxic T lymphotytes (CTLs) which clear virus infection from the CNS. The inability of susceptible mice to clear virus, resulting in virus persistence, was proposed to be from the relative lack of CD8$^+$ CTLs in the CNS during acute infection. In contrast, CD8$^+$ T cells, which are detected during chronic infection in susceptible mice, appear to be important in demyelination, since depletion of these cells in vivo inhibits the extent of myelin destruction (44). However, the specificity of these cells to virus-induced or host antigens is unknown.

Most reports of antiviral CTL activity have involved enveloped viruses which express virus peptides on the membranes of infected cells. The ability of CTLs to lyse

* Corresponding author.

cells infected with nonenveloped viruses (like TMEV) was uncertain. However, studies documenting CTL activity generated against viral nucleoproteins (55) and current concepts of intracytoplasmic antigen processing and association with MHC antigens (6, 14, 28) provide the possibility that CTLs are generated during picornavirus infections. In addition, the presence of anticoxsackievirus CTL in a mouse model of myocarditis (19) provides further strong evidence for CTL involvement in picornavirus infection.

The purpose of this study was to determine whether virus-specific CTLs are generated during TMEV infection and, if present, whether they play a role in resistance/susceptibility. Initially, to determine whether cytolytic lymphocytes are generated during infection, a lectin-dependent cellular cytotoxicity (LDCC) assay was used (2). This assay measures the lytic potential of a lymphocyte population regardless of antigen specificity or MHC restriction (4). Seven days p.i., cytotoxic activity was detected in the CNS of susceptible and resistant TMEV-infected mice. The specificity of this activity was determined by using an anti-TMEV CTL assay. Anti-TMEV CTLs of the $CD8^+$ phenotype were present in TMEV-infected mice of a resistant or susceptible strain 7 days p.i. and was greater in CNS-ILs than in lymphocytes isolated from spleen or lymph node. Further experiments demonstrated the presence of MHC-restricted antiviral CTLs in the CNS of chronically infected SJL/J mice as late as 226 days p.i.

MATERIALS AND METHODS

Virus. The Daniel's strain (10) of TMEV was propagated in BHK-21 cells and used in all experiments. The titer of stock virus used for infection of fibroblast targets was 6.5×10^8 PFU/ml. For infection of mice, TMEV stock was diluted to 2×10^7 PFU/ml, and 0.01 ml of virus was injected into the right cerebral hemisphere.

Mice. C57BL/10SNJ (Jackson Laboratory, Bar Harbor, Maine) or C57BL/6NCr1BR (Charles River, Wilmington, Mass.) (*H-2*^{b}), and SJL/J (Jackson Laboratory) (*H-2*^{s}), were used as prototype resistant and susceptible mouse strains, respectively. B10.S mice were provided by Chella David, director of the Mayo Clinic Immunogenetics Mouse Colony.

Cell culture medium. All cells were grown and assays were performed in RPMI 1640 media supplemented with 200 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 100 U of penicillin, of 100 μg of streptomycin, and 10% fetal bovine serum (FBS; Hyclone, Logan, Utah).

Cell lines. A glioma G26 cell line (*H-2*^{b}) (49) was used for the LDCC assay. Four simian virus 40-transformed cell lines were used for anti-TMEV CTL assays. BLK SVH D.2 A.5R.1 A.3R.1 (*H-2*^{b}) was obtained from the American Type Culture Collection (ATCC TIB 88), whereas C57SV (*H-2*^{b}), PSJLSV (*H-2*^{s}), and BXSF11gSV (*H-2*$^{b/s}$) were kindly provided by Barbara Knowles (Wistar Institute, Philadelphia, Pa.).

Preparation of effectors. For each experiment, mice were intracerebrally inoculated with 2×10^5 PFU of TMEV. At time of termination, brain and spinal cord (CNS) or spleens were placed into 5 ml of RPMI 1640 containing no serum (RPMI-NS). CNS samples were pooled by strain and treatment, while spleens were processed individually.

Splenocytes. Splenocytes were isolated by breaking the spleen capsule and homogenizing the contents with a tissue grinder. The spleen capsule debris was allowed to settle, and the supernatant was placed into a 15-ml centrifuge tube. The pellet was resuspended and allowed to settle in 5 ml of RPMI-NS, and the supernatant was added to the first supernatant. After centrifugation for 10 min ($400 \times g$), the supernatant was discarded and the erythrocytes were lysed with a 4-s treatment with 1 ml of sterile distilled H_2O followed immediately with 14 ml of RPMI-NS. T cells were subsequently enriched by using a nylon wool column (16).

CNS-ILs. Lymphocytes which entered the CNS after virus infection were isolated as described previously (22). Briefly, CNS of infected mice were either minced with a razor blade and further disrupted by vigorous pipetting or homogenized in plastic tubes (100 by 17 mm) with a tissue grinder. Twenty milliliters of the cell suspension was mixed with 10 ml of Percoll (Pharmacia, Uppsala, Sweden) and centrifuged at $27,000 \times g$ for 30 min. The lymphocyte band was removed (22), washed, precleared of erythrocytes with sterile distilled H_2O (as with splenocytes), washed, and counted. The effectors derived from either CNS or spleen were resuspended to 5×10^6 cells per ml, and twofold serial dilutions were made to provide an effector-to-target ratio of 100:1 to 6.25:1.

Preparation of targets. On the day of assay, target cells were removed from the flask by trypsinization, and 1×10^6 to 3×10^6 cells were placed into 15-ml tubes, centrifuged for 10 min ($400 \times g$), and resuspended in 0.2 ml of RPMI-NS. Sodium chromate ($Na_2{}^{51}CrO_4$; New England Nuclear, Boston, Mass.) was added to the cells at 50 μCi/10^6 cells, and the cells were incubated at 37°C for 1 h. After four washes with RPMI–5% FBS, the cells were resuspended to 5×10^4/ml in RPMI–10% FBS and 10^{-4} M 2-mercaptoethanol. ^{51}Cr-labelled glioma G26 targets for the LDCC assay were divided in two; concanavalin A (ConA; 20 μg/ml) was added to one half, whereas the other half was untreated. Targets for the anti-TMEV CTL assay were infected 3 to 24 h prior to trypsinization with an input virus multiplicity of 10 to 15 PFU per cell, which was calculated by trypsinizing and counting cells recovered from a duplicate flask. A third flask was left untreated as an uninfected control. In addition, samples of infected and uninfected target cells were deposited onto glass microscope slides by cytocentrifugation and later examined for presence of virus antigen and class I MHC antigen by the avidin-biotin immunoperoxidase technique as previously described (22).

Rabbit serum containing polyclonal antibody to TMEV, diluted 1:200, was used to detect the presence of viral antigen in TMEV-infected target cells. Class I MHC antigens were detected by using undiluted supernatants of hybridoma M1/42.3.9.8 (ATCC TIB 126), which secretes a rat monoclonal antibody (MAb) specific for all class I haplotypes (48). TMEV-infected and uninfected fibroblasts used in these experiments expressed class I MHC antigens, with a total of 74 to 82% of the TMEV-infected target cells staining positive for virus antigen.

Cytotoxicity assay. One hundred microliters of the target cell suspension was placed in 96-well round-bottom microtiter plates (Costar, Cambridge, Mass.). One hundred microliters of the effector cell suspension was added in triplicate to the targets, resulting in final effector-to-target ratios of 100, 50, 25, 12.5, and 6.25 to 1. Six wells of targets also received medium alone or 10% Triton X-100 detergent (Sigma Chemical, St. Louis, Mo.) to determine spontaneous release or maximum release of chromium from targets, respectively. Plates were incubated for 4 to 6 h at 37°C in 5% CO_2. Half of the cell-free supernatant was removed carefully from each of the wells and assayed in a gamma counter (Beckman Gamma 5500; Beckman Instruments, Irvine, Calif.) for the amount of radioactivity.

TABLE 1. LDCC activity of splenocytes and CNS-ILs from 7-day-TMEV-infected and uninfected C57BL/10SNJ mice

Effector cells	% Specific lysis ± SE with indicated targets[a]					
	Untreated			ConA treated		
	100[b]	50	25	100	50	25
Spleen						
Uninfected mice	2.7 ± 0.0	0.2 ± 0.1	2.1 ± 0.3	5.4 ± 0.3	3.3 ± 0.3	2.4 ± 0.6
Infected mice	3.4 ± 0.8	1.8 ± 0.5	1.1 ± 0.5	11.2 ± 1.1	6.6 ± 0.4	4.3 ± 0.5
CNS-ILs, infected mice	14.2	7.4	5.4	36.1	20.8	12.2

[a] Glioma G26 cells either untreated or ConA treated. Spontaneous release was 18.4% for untreated and 17.3% for ConA-treated targets. Each treatment group contained three mice.
[b] Effector-to-target ratio.

Mean values were calculated from replicate wells, and the results were expressed as percent specific lysis according to the formula [(experimental counts − spontaneous counts)/(maximum counts − spontaneous counts)] × 100.

Percent virus-specific lysis was calculated by subtracting percent specific lysis of uninfected targets from percent specific lysis of infected targets. The percent spontaneous release was calculated by dividing the spontaneous counts by the maximum counts and multiplying the result by 100. The mean spontaneous release averaged (± standard deviation) 26.2% ± 4.9% and 27.4% ± 7.3% for infected and uninfected targets, respectively. These values are similar to those published for other virus models (19, 53). For splenocyte activity, the standard error of the mean was determined from results of multiple mice. For CNS-derived lymphocyte activity, the standard error of the mean was determined from the results obtained from pooled lymphocyte samples in triplicate wells. Statistical comparisons were performed by using the Student *t* test.

In vivo depletion of NK cells. C57BL/6 mice were depleted of natural killer (NK) cells by inoculation of MAb NK1.1 as previously described (36). Briefly, 200 μg of MAb NK1.1 (kindly provided by P. Leibson, Department of Immunology, Mayo Clinic, Rochester, Minn.) was injected intraperitoneally 1 day prior to intracerebral inoculation of TMEV. CNS-ILs were harvested 7 days later and assayed for cytotoxicity.

In vitro blocking of cytotoxicity with MAbs to CD4 and CD8. CNS-ILs were resuspended to 10^7 cells per ml (for a final effector-to-target ratio of 100:1 in RPMI–5% FCS) and placed in round-bottom well plates (Corning) at 50 μl per well. Affinity-purified MAb GK1.5 (ATCC TIB 207; anti-CD4) and MAb 2.43 (ATCC TIB 210; anti-CD8) were diluted to 0.26 and 0.026 mg/ml in RPMI-NS, and 50 μl was added to appropriate wells. Control wells received 50 μl of RPMI-NS with no antibody. After incubation at 4°C for 30 to 45 min, 100 μl of ^{51}Cr-labelled targets was added to appropriate wells.

RESULTS

Cytotoxic activity in lymphocytes isolated from TMEV-infected mice. The ability of TMEV-infected mice to generate a cytotoxic lymphocyte response was determined initially by using the LDCC assay, which measures the cytotoxic potential in a population of lymphocytes irrespective of antigen specificity or MHC restriction (2, 4). For T cells, the lectin (ConA) aids in increasing the proximity of effector and target through interaction of class I MHC products (15) and the T-cell receptor (18). Cytotoxic activity was examined initially in resistant C57BL/10SNJ mice because these mice had been shown to respond to TMEV infection in a typical monophasic response (22), with an increase in $CD8^+$ T cells infiltrating the CNS (22) and subsequent clearance of virus (36, 42). Nylon wool-nonadherent spleen cells from TMEV-infected C57BL/10SNJ mice 7 days p.i. displayed greater LDCC activity than did spleen cells from uninfected mice (Table 1). However, CNS-ILs of infected mice demonstrated considerably greater cytotoxic activity than did splenocytes. Nonspecific lysis of untreated targets suggested the presence of NK cells in the CNS-infiltrating lymphocyte population. The extent of LDCC activity in CNS-ILs from uninfected mice could not be determined because very few lymphocytes were present in the CNS of these mice. These results indicated cytotoxic activity in CNS-ILs of TMEV-infected mice.

Generation of cytotoxic lymphocytes in mice depleted of NK cells. To determine the role of NK cells in the LDCC activity induced by TMEV infection, C57BL/10SNJ mice were depleted in vivo of NK cells by intraperitoneal injection of 200 μg of MAb NK1.1 1 day prior to intracerebral TMEV inoculation. CNS-ILs isolated from control phosphate-buffered saline (PBS)-treated mice (7 days p.i.) displayed cytolytic activity against ConA-treated targets and low levels of nonspecific activity against untreated targets (Fig. 1). Although reduced, CNS-ILs from NK1.1-treated mice nevertheless lysed ConA-treated targets (44% [PBS treated] versus 28% [NK1.1 treated]) at a 50:1 ratio. NK1.1-treated mice were depleted of NK cells, since nonspecific lysis of untreated targets no longer was detected. Therefore, even though NK cells did account for some of the cytotoxic activity in the CNS of TMEV-infected mice, T cells ($NK1.1^-$) were responsible for the majority of the LDCC activity.

Comparison of LDCC activity in mice resistant or susceptible to TMEV-induced demyelination. To determine whether the mechanism of resistance to demyelination is in part due to the presence of CTLs in the CNS, LDCC activity was compared between C57BL/10SNJ mice (resistant) and SJL/J mice (susceptible). LDCC activity in C57BL/10SNJ mice was greater than in SJL/J mice (44% versus 23% specific lysis at a 50:1 effector-to-target ratio) (Fig. 2). Since SJL/J mice differ from C57BL/10SNJ mice not only in MHC genes but also in other background genes, the experiment was repeated to compare C57BL/10SNJ [*H-2*^{b}] and B10.S [*H-2*^{s}] congeneic mice, which differ only in the MHC region. Results from this comparison showed no difference in LDCC activity (16.1% [B10.S] versus 12.7% [C57BL/10] at a 50:1 effector-to-target ratio). These results indicated that although the effectors of LDCC activity may play a role in resistance to TMEV infection, they are not the only factor because some strains that develop virus persistence and

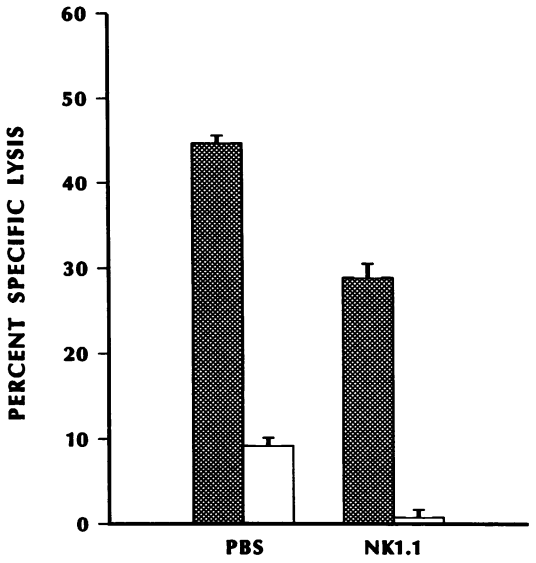

FIG. 1. LDCC activity in CNS-ILs 7 days p.i. in untreated and NK1.1-treated C57BL/10SNJ mice. Cytotoxicity was assayed against G26 glioma cells with (gray bars) and without (open bars) ConA. Results represent the pooled activity of CNS-ILs isolated from three mice in each treatment group. Percent spontaneous release was 17.7% for ConA-treated targets and 17.0% for untreated targets. NK1.1 treatment reduced significantly the lysis of ConA-treated targets ($P = 0.01$) and untreated targets ($P < 0.01$).

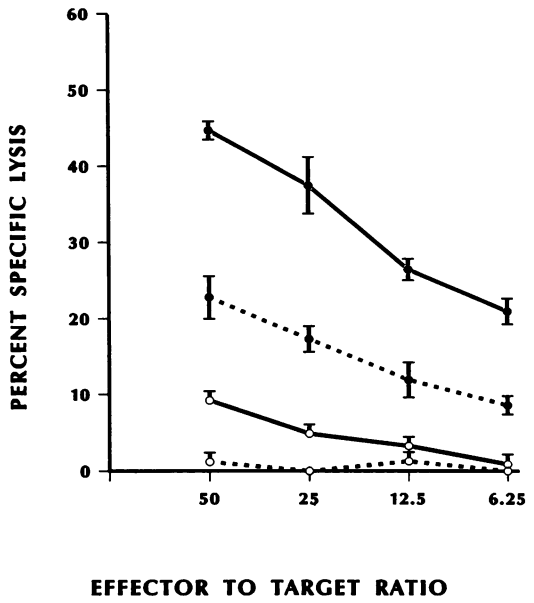

FIG. 2. Comparison of LDCC activity between resistant C57BL/10SNJ (solid lines) and susceptible SJL/J (dashed lines) mice. Cytotoxicity was assayed against G26 glioma cells with (filled circles) and without (open circles) ConA. Results represent the pooled activity of CNS-ILs isolated from three C57BL/10SNJ and three SJL/J mice. Percent spontaneous release was 17.7% for ConA-treated targets and 17.0% for untreated targets. Lysis by CNS-ILs from SJL/J mice was significantly less than lysis by CNS-ILs from C57BL/10SNJ mice against ConA-treated targets ($P = 0.015$) and untreated targets ($P = 0.01$).

subsequent demyelination also generate cytotoxic effectors as detected by LDCC.

Anti-TMEV cytotoxic activity in the CNS of infected C57BL/10SNJ mice. Since maximum cytolytic activity as measured by LDCC was detected from lymphocytes isolated from the CNS 7 days following infection of resistant mice, this experimental paradigm was used initially to examine antiviral cytotoxic activity. Simian virus 40-transformed cell lines derived from histocompatible mice were used as targets. To determine the optimal length of in vitro infection, target cells were inoculated with input virus of 10 PFU per cell for 3, 5, or 20 h. TMEV-specific cytotoxic activity was present in the CNS-ILs of 7-day-infected C57BL/6 mice (Fig. 3). The best targets for detecting anti-TMEV cytotoxic activity were those infected for 20 h prior to assay. These targets had spontaneous release comparable to that of targets infected for shorter periods of time (24.7% [20 h] versus 31.2% [5 h] versus 29.8% [3 h]).

Comparisons of anti-TMEV cytotoxic activity between resistant and susceptible mouse strains and of MHC restriction. Comparable levels of anti-TMEV cytotoxic activity were observed in CNS-ILs from C57BL/6 and SJL/J mice (Fig. 4) 7 days p.i. CNS-ILs from either strain lysed infected targets only, and they were MHC-restricted, since lysis was detected only when we used targets that were matched for *H-2* with their respective effectors. To eliminate the possibility of target differences in virus replication and antigen presentation, simian virus 40-transformed fibroblasts derived from (B6 × SJL)F_1 mice, which express both $H\text{-}2^b$ and $H\text{-}2^s$ MHC antigens, were used. These targets when infected with TMEV were lysed at comparable levels by the CNS-IL population from SJL/J or C57BL/6 mice (32.2% [SJL] versus 23.1% [C57BL/6] at a 100:1 effector-to-target ratio).

Comparison of anti-TMEV cytotoxic activity between CNS-ILs and lymphocytes isolated from other organs. To determine the extent of anti-TMEV activity present in other immune organs, we compared cytotoxic activity in CNS-ILs, splenocytes, and lymph node cells. In SJL/J mice, CNS-ILs showed greater CTL activity than did nylon wool-treated spleen cells (29.2% ± 2.3% [CNS-ILs] versus 2.9% ± 0.9% [spleen cells]; $P < 0.01$). Similarly, CNS-ILs from C57BL/10SNJ mice showed greater anti-TMEV cytotoxic activity than did lymphocytes from cervical lymph nodes (46.2% ± 5.3% [CNS-ILs] versus 2.8% ± 0.9% [lymph node cells]; $P < 0.01$).

Extent of NK cell involvement in the anti-TMEV cytotoxic response. Lysis of uninfected *H-2*-matched fibroblasts was significantly greater in CNS-ILs from resistant C57BL/10SNJ mice than in those from SJL/J mice (13.1% ± 4.1% versus 0.8% ± 0.3% at a 100:1 effector-to-target ratio; $P = 0.024$). This nonspecific lytic activity suggested the presence of NK cells in the CNS-IL population. To determine the extent of NK involvement in the anti-TMEV cytotoxic response, C57BL/10SNJ mice were depleted of NK cells in vivo by using MAb NK1.1 1 day before TMEV infection. In lymphocytes isolated from CNS 7 days p.i., anti-TMEV cytotoxic activity against uninfected targets decreased (22.1% [untreated] versus 8.5% [NK1.1 treated]), whereas specific lysis of infected targets remained the same (55% [untreated] versus 50% [NK1.1 treated]) (Fig. 5).

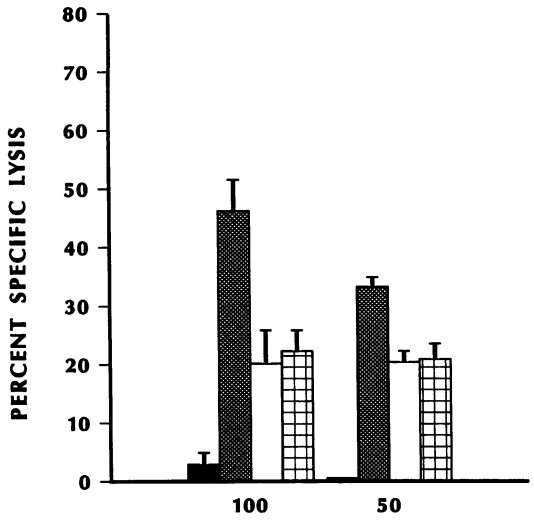

FIG. 3. Effect of length of infection of targets on anti-TMEV cytotoxicity. Targets were BLK SVH D.2 A.5R.1 A.3R.1 fibroblast cells infected for 20 h (gray bars), 5 h (open bars), or 3 h (checked bars) or uninfected (black bars). Results represent the pooled activity of CNS-ILs isolated from 15 C57BL/6 mice. Percent spontaneous release was 24.7% for 20-h-infected targets, 31.2% for 5-h-infected targets, 29.8% for 3-h-infected targets, and 26.3% for uninfected targets. Lysis of 20-h-infected targets was significantly greater than lysis of 5-h-infected targets ($P < 0.01$) or 3-h-infected targets ($P = 0.02$) at an effector-to-target ratio of 50:1.

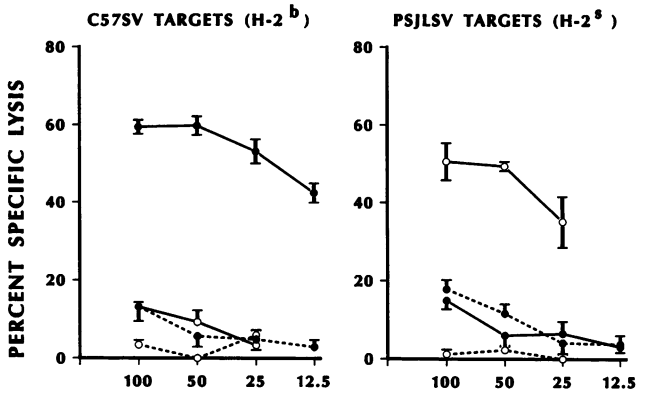

FIG. 4. Comparison of anti-TMEV cytotoxic activity from CNS lymphocytes isolated from resistant C57BL/6 (filled circles) or susceptible SJL/J (open circles) mice. Cytotoxicity was assayed against TMEV-infected (solid lines) or uninfected (dashed lines) C57SV (*H-2*^{b}) and PSJLSV (*H-2*^{s}) fibroblast targets. Results represent the pooled activity of CNS-ILs isolated from 5 C57BL/6 and 10 SJL/J mice. Percent spontaneous release was 36.5% for infected and 30.1% for uninfected C57SV targets and 33.8% for infected and 28.4% for uninfected PSJLSV targets.

These results provided strong evidence that T cells are responsible primarily for the lysis of TMEV-infected targets, whereas NK cells are important in the lysis of noninfected targets.

Characterization of T cells responsible for anti-TMEV cytotoxic activity, using in vitro blocking with an anti-CD8 or anti-CD4 MAb. MHC restriction of anti-TMEV cytotoxic activity and the presence of anti-TMEV activity in NK1.1-treated and infected C57BL/10SNJ mice provided evidence that cytotoxic T cells were present in the CNS 7 days p.i. To characterize further the cells responsible for anti-TMEV cytotoxicity, CNS-ILs from SJL/J mice and NK1.1-pretreated C57BL/10SNJ mice were reacted in vitro with an anti-CD8 or anti-CD4 MAb prior to the addition of ^{51}Cr-labelled *H-2*-matched targets. Addition of these antibodies to the assay inhibits the function of the respective cell types, causing a decrease in lymphocyte activity (30, 31). Anti-TMEV activity in lymphocytes isolated from NK1.1-pretreated C57BL/10SNJ mice and SJL/J mice was inhibited almost completely by addition of the anti-CD8 MAb (Fig. 6 and 7), whereas no effect was seen with addition of the anti-CD4 MAb. However, it was not possible to exclude the presence of CD4$^+$ CTLs, since the targets used in these experiments did not express detectable levels of class II MHC antigens by the immunoperoxidase technique.

Detection of anti-TMEV CTLs in CNS-ILs from chronically infected mice. CNS-ILs from susceptible SJL/J mice infected chronically (36, 49, and 226 days) with TMEV were assayed for the presence of anti-TMEV CTLs. Antiviral cytotoxic activity was detected as late as 226 days p.i. (Fig. 8). The cytotoxic activity was *H-2* restricted because CNS-ILs were unable to lyse mismatched targets. Lysis of uninfected targets was also not detected in these experiments. These results provide evidence that anti-TMEV CTLs are present in the CNS of chronically infected SJL/J mice and suggest

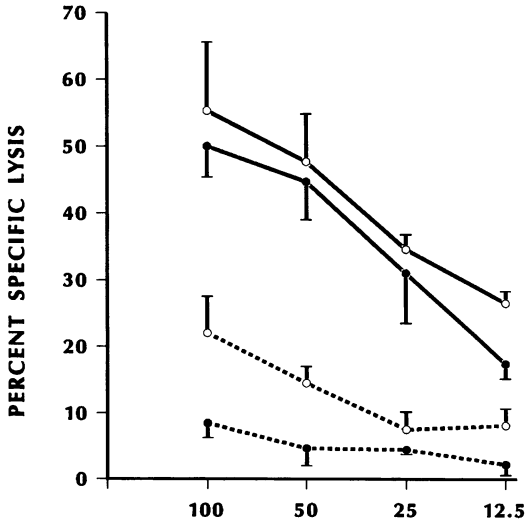

FIG. 5. Effect of in vivo depletion of NK cells on antiviral cytotoxicity. Anti-TMEV cytotoxic activity was assayed in CNS-ILs 7 days p.i. in untreated (open circles) and NK1.1-treated (closed circles) C57BL/10SNJ mice, using TMEV-infected (solid lines) and uninfected (dashed lines) C57SV (*H-2*^{b}) fibroblast targets. Results represent the pooled activity of CNS-ILs isolated from 10 NK1.1-treated and 8 PBS-treated mice. Percent spontaneous release was 25.8% for infected targets and 32.2% for uninfected targets.

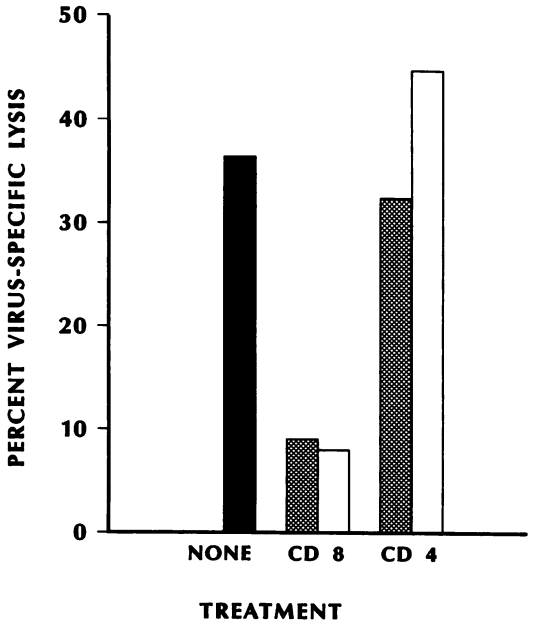

FIG. 6. Effect of anti-CD4 or anti-CD8 in vitro treatment on anti-TMEV cytotoxic activity of CNS lymphocytes from NK1.1-pretreated C57BL/10SNJ mice 7 days p.i. Cytotoxicity was assayed without antibody (black bar) and in the presence of antibodies at two concentrations (0.26 μg/ml [gray bars] and 0.026 μg/ml [open bars]) at an effector-to-target ratio of 100:1. Results are expressed as virus-specific lysis, which equals percent specific lysis against TMEV-infected C57SV ($H\text{-}2^b$) fibroblasts minus percent specific lysis against uninfected fibroblasts. Results represent the pooled activity of CNS-ILs isolated from 30 mice. Percent spontaneous release was 30.9% for infected targets and 44.1% for uninfected targets.

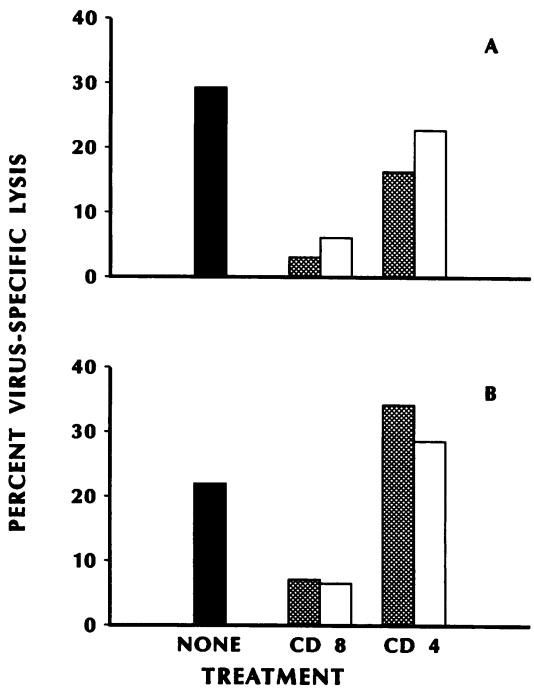

FIG. 7. Effect of anti-CD4 and anti-CD8 in vitro treatment on anti-TMEV cytotoxic activity on CNS lymphocytes isolated from SJL/J mice 7 days p.i. Cytotoxicity was assayed without antibody (black bar) and in the presence of antibodies at two concentrations (0.26 μg/ml [gray bars] and 0.026 μg/ml [open bars]). Results are expressed as virus-specific lysis. Results are from two separate experiments (A and B) and represent the pooled activity of CNS-ILs isolated from 20 mice for each experiment. Percent spontaneous release was 24.1% for infected targets and 28.6% for uninfected targets in experiment A and was 26.4% for infected targets and 18.4% for uninfected targets in experiment B.

that these cells may play a role in lysis of TMEV-infected glial cells, resulting in demyelination.

DISCUSSION

These experiments demonstrated the presence of $CD8^+$ *H-2*-restricted virus-specific CTLs in the CNS of mice infected with TMEV. Of interest, TMEV-specific CTL activity was similar during the early infection of mice resistant or susceptible to TMEV-induced demyelination. However, mice which are susceptible to demyelination showed antiviral cytotoxic activity in CNS as late as 226 days p.i. Lymphocytes from spleens or lymph nodes of infected mice displayed less cytotoxic activity. The presence of the lytic activity primarily in the CNS-IL population is consistent with the fact that the CNS is the target organ for this virus following intracerebral inoculation (50). Similarly, CTLs to hepatitis A virus have been detected almost exclusively in liver biopsies but not in blood of hepatitis A virus-infected patients (52). The inability of CNS-ILs to lyse mismatched targets and the persistence of the cytotoxic response following depletion of NK cells provided further evidence that the cytotoxic lymphocytes are of T-cell origin. The reproducibility in demonstrating this cytotoxic activity in seven of eight (C57BL/10SNJ) and five of five (SJL/J) of our experiments provides strong evidence that virus-specific CTLs are generated during TMEV infection.

In vitro blocking studies using MAbs to T-cell subsets determined that the antiviral cytotoxic response in both resistant and susceptible mice was mediated predominantly by $CD8^+$ T lymphocytes. The greater sensitivity of the chromium release assay enabled us to detect $CD8^+$ lymphocytes in the CNS early in the immune response of SJL/J mice even though the cells were not detected previously by immunohistochemical methods (22). We were not, however, able to rule out the presence of $CD4^+$ CTLs in the CNS, since our target cells did not express detectable levels of class II MHC antigens.

The early generation of anti-TMEV CTLs by SJL/J mice suggests that mice susceptible to TMEV-induced demyelination have the capacity to eliminate virus infection in a manner similar to that of resistant mice. CTLs must recognize virus-infected cells in association with MHC glycoproteins expressed on the cell surface. Although cells of the CNS are typically void of MHC antigens (11, 54), glial cells express MHC antigens following virus infection (23, 33), during autoimmune demyelination (29), and in multiple sclerosis (51). However, the ability of neurons to express MHC antigens is uncertain. The fact that TMEV primarily infects neurons during early infection raises the possibility that the early generation of antiviral CTLs may be of less importance during acute disease than during chronic demyelinating disease, when virus antigens and class I MHC antigens are expressed on oligodendrocytes, astrocytes, and macrophages (9, 41, 47).

The differences in pathology observed when one compares

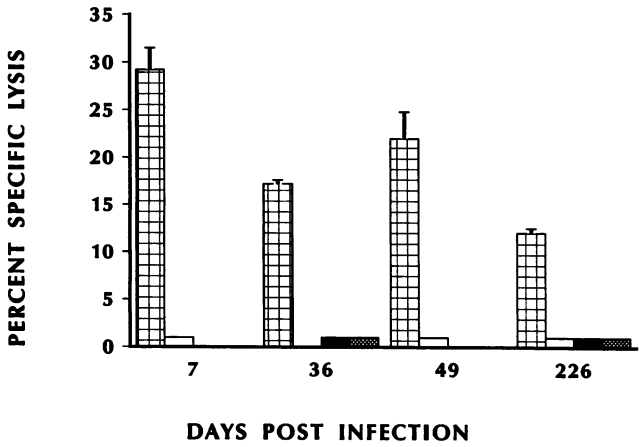

FIG. 8. Anti-TMEV CTL activity of CNS-ILs from chronically infected SJL/J (*H-2*^{s}) mice. CNS-ILs were isolated from SJL/J mice infected with TMEV for 7, 36, 49, and 226 days. Targets were TMEV infected (checked bars) and uninfected (open bars) PSJLSV (*H-2*^{s}) fibroblasts and TMEV-infected (black bars) and uninfected (gray bars) C57SV (*H-2*^{b}) fibroblasts. The results shown are from two separate experiments comparing day 7 with day 49 p.i. and comparing day 36 with day 226. Results represent pooled activity of CNS-ILs isolated from 10 mice for each time point. Percent spontaneous release for the day 7 and day 49 experiments was 24.2% for infected and 18.9% for uninfected PSJLSV (*H-2*^{s}) targets. Percent spontaneous release for the day 36 and day 226 experiment was 26.4% for infected and 30.1% for uninfected PSJLSV (*H-2*^{s}) targets and 24.4% for infected and 26.9% for uninfected C57SV (*H-2*^{b}) targets.

infection of resistant and susceptible mice may be in antigen presentation, since appropriate MHC antigens may not be expressed in specific brain cells during the early phase of disease. Mouse hepatitis virus has been shown to down-regulate MHC expression of H-2K antigens following in vitro infection of endothelial cells (20). Preliminary data from our laboratory suggest differential expression in vivo of class I MHC antigens in cells infected with TMEV.

Alternatively, the NK cell response may be more important in limiting the amount of virus replication in TMEV-infected neurons, since NK cells are more active against target cells that lack MHC antigens (27). NK cells were responsible for some of the cytotoxic activity, as demonstrated by the lysis of uninfected targets. Although CNS-ILs from TMEV-infected SJL/J mice rarely lysed *H-2*-matched-uninfected targets, CNS-ILs from infected C57BL/10SNJ mice lysed uninfected targets by as much as 20 to 50% of the levels observed against TMEV-infected targets (four of seven experiments). This nonspecific lysis was diminished by depletion of NK cells in C57BL/10SNJ mice prior to TMEV infection, with minimal effect on cytotoxicity of TMEV-infected targets. Of the strains used in this study, C57BL/10SNJ mice are considered normal NK responders, while SJL/J mice are low NK responders (5, 7, 17, 21). Because of a difference in NK cell function, SJL/J mice may be unable to limit early virus infection. This may allow for subsequent infection of oligodendrocytes that may be lysed during chronic infection by previously primed anti-TMEV CTLs. In support of this hypothesis, we have observed that the number of TMEV-infected cells in the brain peaks by day 4 p.i. in C57BL/10SNJ mice, whereas the number of TMEV-infected cells increases through day 7 p.i. in SJL/J mice. In addition, depletion of NK cells in resistant mice (36) results in increased frequency of severe encephalitis, neuronal necrosis in anterior horn cells, and demyelination in spinal cord.

Finally, our results demonstrate that MHC-restricted anti-TMEV CTLs are present in the CNS of susceptible SJL/J mice as late as 226 days p.i. The presence of anti-TMEV CTLs in the CNS of chronically infected animals provides further evidence that demyelination is in part mediated by CD8$^+$ *H-2*-restricted T cells. The role of CD8$^+$ lymphocytes in enhancing pathology in virus infections of the CNS has been described in other viral models, including infection with lymphocytic choriomeningitis virus (LCMV). LCMV infection in neonatal or immunosuppressed mice results in asymptomatic infection and virus persistence (13). Adoptive transfer of LCMV-specific CD8$^+$ CTL clones into these mice results in increased pathology and death (3, 12, 13). The disease induced by Semliki Forest virus is also immune mediated and has been proposed to be mediated by antiviral CTLs (34). The persistence of TMEV in the presence of anti-TMEV CTLs provides an interesting paradox. Recently, it has been demonstrated by using the LCMV model that virus mutants arise during infection that can evade antiviral CTLs (1, 32, 37). As LCMV and TMEV are both RNA viruses with high mutation rates, it is possible that a similar scenario may occur with TMEV infection. Further efforts to determine the antigen specificity of the anti-TMEV CTL response during demyelination will be critical for understanding the mechanism of virus-induced CNS demyelination.

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REFERENCES

1. **Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. A. Oldstone.** 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. J. Exp. Med. **60**:521–540.
2. **Asherson, G. L., J. Ferluga, and G. Janossy.** 1973. Non-specific cytotoxicity by T cells activated with plant mitogens *in vitro* and the requirement for plant agents during the killing reaction. Clin. Exp. Immunol. **15**:573–589.
3. **Baenziger, J., H. Hengartner, R. M. Zinkernagel, and G. A. Cole.** 1986. Induction or prevention of immunopathological disease by cloned cytotoxic T cell lines specific for lymphocytic choriomeningitis virus. Eur. J. Immunol. **16**:387–393.
4. **Bevan, M. J., and M. Cohn.** 1975. Cytotoxic effects of antigen- and mitogen-induced T cells on various targets. J. Immunol. **114**:559–565.
5. **Blair, P. B.** 1980. Immunogenetics of natural immunity, p. 401–409. *In* R. B. Herberman (ed.), Natural cell-mediated immunity against tumors. Academic Press, Inc., New York.
6. **Braciale, T. J., L. A. Morrison, M. T. Sweetser, J. Sambrook, M.-J. Gething, and V. L. Braciale.** 1987. Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. Immunol. Rev. **98**:95–114.
7. **Clark, E. A., N. T. Windsor, J. C. Sturge, and T. H. Stanton.** 1980. Mutations that influence natural cell-mediated cytotoxicity in rodents, p. 417–429. *In* R. B. Herberman (ed.), Natural cell-mediated immunity against tumors. Academic Press, Inc., New York.
8. **Clatch, R. J., R. W. Melvold, S. D. Miller, and H. L. Lipton.** 1985. Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease in mice is influenced by the H-2D

region: correlation with TMEV-specific delayed-type hypersensitivity. J. Immunol. **135**:1408–1414.
9. **Dal Canto, M. C., and H. L. Lipton.** 1982. Ultrastructural immunohistochemical localization of virus in acute and chronic demyelinating Theiler's virus infection. Am. J. Pathol. **106**:20–29.
10. **Daniels, J. B., A. M. Pappenheimer, and S. Richardson.** 1952. Observations on encephalomyelitis of mice (DA strain). J. Exp. Med. **96**:517–530.
11. **Date, I., K. Kawamura, and H. Nakashima.** 1988. Histological signs of immune reactions against allogeneic solid fetal neural grafts in the mouse cerebellum depend on the MHC locus. Exp. Brain Res. **73**:15–22.
12. **Dixon, J. E., J. E. Allan, and P. C. Doherty.** 1987. The acute inflammatory process in murine lymphocytic choriomeningitis is dependent on Lyt-2^+ immune T cells. Cell. Immunol. **107**: 8–14.
13. **Doherty, P. C., J. E. Allan, F. Lynch, and R. Ceredig.** 1990. Dissection of an inflammatory process induced by CD8+ T cells. Immunol. Today **11**:55–59.
14. **Germain, R. N.** 1986. The ins and outs of antigen processing and presentation. Nature (London) **322**:687–689.
15. **Gorman, K. C., K. P. Kane, and W. R. Clark.** 1987. Target cell recognition structures in LDCC and ODCC. J. Immunol. **138**: 1014–1019.
16. **Henry, C.** 1980. Cell separation: nylon wool, p. 182–185. *In* B. B. Mischell and S. M. Shiigi (ed.), Selected methods in cellular immunology. W. H. Freeman and Co., New York.
17. **Herberman, R. B.** 1980. Summary: immunogenetic regulation of NK activity, p. 443–447. *In* R. B. Herberman (ed.), Natural cell-mediated immunity against tumors. Academic Press, Inc., New York.
18. **Hubbard, S. C., D. M. Kranz, G. D. Longmore, M. V. Sitkovsky, and H. N. Eisen.** 1986. Glycosylation of the T-cell antigen-specific receptor and its potential role in lectin-mediated cytotoxicity. Proc. Natl. Acad. Sci. USA **83**:1852–1856.
19. **Huber, S. A., L. P. Job, and J. F. Woodruff.** 1984. In vitro culture of coxsackievirus group B, type 3 immune spleen cells on infected endothelial cells and biological activity of the cultured cells in vivo. Infect. Immun. **43**:567–573.
20. **Joseph, J., R. L. Knobler, F. D. Lublin, and M. N. Hart.** 1989. Differential modulation of MHC class I antigen expression on mouse brain endothelial cells by MHV-4 infection. J. Neuroimmunol. **22**:241–253.
21. **Kaminsky, S. G., V. Milisauskas, P. B. Chen, and I. Nakamura.** 1987. Defective differentiation of natural killer cells in SJL mice. Role of the thymus. J. Immunol. **138**:1020–1025.
22. **Lindsley, M. D., and M. Rodriguez.** 1989. Characterization of the inflammatory response in the central nervous system of mice susceptible or resistant to demyelination by Theiler's virus. J. Immunol. **142**:2677–2682.
23. **Lindsley, M. D., R. L. Thiemann, and M. Rodriguez.** 1988. Expression of class I antigen in C57BL/10 and SJL mice following Theiler's virus infection. J. Neuropathol. Exp. Neurol. **47**:324.
24. **Lipton, H. L.** 1975. Theiler's virus infection in mice: an unusual biphasic disease process leading to demyelination. Infect. Immun. **11**:1147–1155.
25. **Lipton, H. L., and M. C. Dal Canto.** 1976. Theiler's virus-induced demyelination: prevention by immunosuppression. Science **192**:62–64.
26. **Lipton, H. L., and M. C. Dal Canto.** 1977. Contrasting effects of immunosuppression on Theiler's virus infection in mice. Infect. Immun. **15**:903–909.
27. **Ljunggren, H.-G., and K. Kärre.** 1990. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol. Today **11**:237–244.
28. **Long, E. O., and S. Jacobson.** 1989. Pathways of viral antigen processing and presentation to CTL: defined by the mode of virus entry? Immunol. Today **10**:45–48.
29. **Matsumoto, Y., and M. Fujiwara.** 1986. In situ detection of class I and II major histocompatibility complex antigens in the rat central nervous system during experimental allergic encephalomyelitis. An immunohistochemical study. J. Neuroimmunol. **12**:265–277.
30. **Meuer, S. C., R. E. Hussey, J. C. Hodgdon, T. Hercend, S. F. Schlossman, and E. L. Reinherz.** 1982. Surface structures involved in target recognition by human cytotoxic T lymphocytes. Science **218**:471–473.
31. **Meuer, S. C., S. F. Schlossman, and E. L. Reinherz.** 1982. Clonal analysis of human cytotoxic T lymphocytes: $T4^+$ and $T8^+$ effector T cells recognize products of different major histocompatibility complex regions. Proc. Natl. Acad. Sci. USA **79**:4395–4399.
32. **Oldstone, M. B. A., M. Salvato, A. Tishon, and H. Lewicki.** 1988. Virus-lymphocyte interactions. III. Biologic parameters of a virus variant that fails to generate CTL and establishes persistent infection in immunocompetent hosts. Virology **164**:507–516.
33. **Olsson, T., J. Maehlen, A. Löve, L. Klareskog, E. Norrby, and K. Kristensson.** 1987. Induction of class I and class II transplantation antigens in rat brain during fatal and non-fatal measles virus infection. J. Neuroimmunol. **16**:215–224.
34. **Pathak, S., S. J. Illavia, and H. E. Webb.** 1983. The identification and role of cells involved in CNS demyelination in mice after Semliki forest virus infection: an ultrastructural study. Prog. Brain Res. **59**:237–254.
35. **Patick, A. K., L. R. Pease, C. S. David, and M. Rodriguez.** 1990. Major histocompatibility complex-conferred resistance to Theiler's virus-induced demyelinating disease is inherited as a dominant trait in B10 congenic mice. J. Virol. **64**:5570–5576.
36. **Paya, C. V., A. K. Patick, P. J. Leibson, and M. Rodriguez.** 1989. Role of natural killer cells as immune effectors in encephalitis and demyelination induced by Theiler's virus. J. Immunol. **143**:95–102.
37. **Pircher, H., D. Moskophidis, U. Rohrer, K. Bürki, H. Hengartner, and R. M. Zinkernagel.** 1990. Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo. Nature (London) **346**:629–633.
38. **Rodriguez, M., and C. S. David.** 1985. Demyelination induced by Theiler's virus: influence of the H-2 haplotype. J. Immunol. **135**:2145–2148.
39. **Rodriguez, M., W. P. Lafuse, J. Leibowitz, and C. S. David.** 1986. Partial suppression of Theiler's virus-induced demyelination in vivo by administration of monoclonal antibodies to immune-response gene products (Ia antigens). Neurology **36**: 964–970.
40. **Rodriguez, M., J. Leibowitz, and C. S. David.** 1986. Susceptibility to Theiler's virus-induced demyelination. Mapping of the gene within the H-2D region. J. Exp. Med. **163**:620–631.
41. **Rodriguez, M., J. L. Leibowitz, and P. W. Lampert.** 1983. Persistent infection of oligodendrocytes in Theiler's virus-induced encephalomyelitis. Ann. Neurol. **13**:426–433.
42. **Rodriguez, M., A. K. Patick, and L. R. Pease.** 1990. Abrogation of resistance to Theiler's virus-induced demyelination in C57BL mice by total body irradiation. J. Neuroimmunol. **26**:189–199.
43. **Rodriguez, M., and J. Quddus.** 1986. Effect of cyclosporin A, silica quartz dust, and protease inhibitors on virus-induced demyelination. J. Neuroimmunol. **13**:159–174.
44. **Rodriguez, M., and S. Sriram.** 1988. Successful therapy of Theiler's virus-induced demyelination (DA strain) with monoclonal anti-Lyt-2 antibody. J. Immunol. **140**:2950–2955.
45. **Roos, R. P., and R. Wollmann.** 1984. DA strain of Theiler's murine encephalomyelitis virus induces demyelination in nude mice. Ann. Neurol. **15**:494–499.
46. **Rosenthal, A., R. S. Fujinami, and P. W. Lampert.** 1986. Mechanism of Theiler's virus-induced demyelination in nude mice. Lab. Invest. **54**:515–522.
47. **Sethi, P., and H. L. Lipton.** 1983. Location and distribution of virus antigen in the central nervous system of mice persistently infected with Theiler's virus. Br. J. Exp. Pathol. **64**:57–65.
48. **Stallcup, K. C., T. A. Springer, and M. F. Mescher.** 1981. Characterization of an anti-H-2 monoclonal antibody and its use in large-scale antigen purification. J. Immunol. **127**:923–930.
49. **Sundarraj, N., M. Schachner, and S. E. Pfeiffer.** 1975. Biochemically differentiated mouse glial lines carrying a nervous system

specific cell surface antigen (NS-1). Proc. Natl. Acad. Sci. USA **72**:1927–1931.
50. **Theiler, M.** 1937. Spontaneous encephalomyelitis of mice, a new virus disease. J. Exp. Med. **65**:705–719.
51. **Traugott, U.** 1987. Multiple sclerosis: relevance of class I and class II MHC-expressing cells to lesion development. J. Neuroimmunol. **16**:283–302.
52. **Vallbracht, A., K. Maier, Y.-D. Stierhof, K. H. Wiedmann, B. Flehmig, and B. Fleischer.** 1989. Liver-derived cytotoxic T cells in hepatitis A virus infection. J. Infect. Dis. **160**:209–217.
53. **Welsh, R. M., Jr., R. M. Zinkernagel, and L. A. Hallenbeck.** 1979. Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice. II. "Specificities" of the natural killer cells. J. Immunol. **122**:475–481.
54. **Wong, G. H. W., P. F. Bartlett, I. Clark-Lewis, F. Battye, and J. W. Schrader.** 1984. Inducible expression of H-2 and Ia antigens on brain cells. Nature (London) **310**:688–691.
55. **Wraith, D. C.** 1987. The recognition of influenza A virus-infected cells by cytotoxic T lymphocytes. Immunol. Today **8**:239–246.