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Role of T cells in resistance to Theiler's virus infection

Moses Rodriguez,* Mark D. Lindsley and Mabel L. Pierce

Departments of Neurology and Immunology, Mayo Clinic and Mayo Foundation, Rochester, MN 55905, U.S.A.

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Intracerebral infection of C57BL/10SNJ mice with Theiler's virus results in acute encephalitis with subsequent virus clearance and absence of spinal cord demyelination. In contrast, infection of SJL/J mice results in acute encephalitis, virus persistence, and immune-mediated demyelination. These experiments examined the role of T-cell subsets in the *in vivo* immune response to Theiler's virus in resistant C57BL/10SNJ mice. Depletion of T-cell subsets with monoclonal antibodies (mAbs) directed at CD3 (pan-T-cell marker), $CD4^+$ (class II-restricted) or $CD8^+$ (class I-restricted) T cells resulted in increased frequency of paralysis and death as a result of acute encephalitis. Neuropathologic studies 10 days after infection demonstrated prominent necrosis, primarily in the pyramidal layer of hippocampus and in the thalamus of mice depleted of T-cell subsets. In immunosuppressed and infected C57BL/10SNJ mice, analysis of spinal cord sections 35 days after infection demonstrated small demyelinated lesions relatively devoid of inflammatory cells even though virus antigen could be detected by immunocytochemistry. Both $CD4^+$ and $CD8^+$ T cells are important in the resistance to infection with Theiler's virus in C57BL/10SNJ mice. However, subsequent spinal cord demyelination, to the extent observed in susceptible mice, depends on the presence of virus antigen persistence and a competent cellular immune response.

Key words: cytotoxic T cell; multiple sclerosis; myelin.

Introduction

Intracerebral infection of C57BL/10SNJ mice with Theiler's murine encephalomyelitis virus (TMEV), a picornavirus, results in acute encephalitis characterized by infiltrates of inflammatory cells within the meninges, hippocampus, thalamus, hypothalamus, and, to a lesser extent, cerebral cortex within 3 to 7 days of infection.[1] The mononuclear inflammatory infiltrate consists of $CD8^+$ and $CD4^+$ T cells, macrophages, B cells, and occasional plasma cells in a typical monophasic immune response.[2] Virus antigen is detected primarily within neurons in the deep structures of the brain, i.e. pyramidal neurons of the hippocampus, thalamus, hypothalamus, striatum, temporal lobes of the cerebral cortex, and occasionally in the anterior horn cells of the spinal cord.[3] Virus titers from the central nervous system (CNS) peak on day 7 but infection is cleared rapidly in most mice within 2 to 3 weeks of infection.[3] These mice show no neurologic abnormalities during this early infection and clinical or pathologic signs of chronic demyelination do not develop in the spinal cord.

In contrast, intracerebral infection of susceptible SJL/J mice with TMEV causes a

* Author to whom reprint requests should be addressed at: Departments of Neurology and Immunology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, U.S.A.

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biphasic disease characterized by acute encephalitis followed by chronic demyelination of spinal cord.[4,5] Virus antigen is detected in neurons of the brain during the acute disease, but oligodendrocytes, astrocytes, and macrophages express virus antigen and virus RNA in the spinal cord during the chronic phase of disease.[6–8] Infectious virus can be detected in the CNS throughout the course of the infection for as long as 1 to 2 years.[9] An intense inflammatory response consisting primarily of $CD8^+$ and $CD4^+$ T cells persists primarily in the spinal cord.[2] The pathologic condition induced by chronic persistent infection is similar to multiple sclerosis, thus making this an excellent animal model of human virus-induced demyelinating disorders.[10]

The reason for the difference between susceptibility and resistance to TMEV-induced chronic demyelinating disease is unknown, but there is evidence that the immune response plays a role. C57BL/10SNJ congenic mice of *b*, *d*, or *k* haplotypes are able to clear virus infection and demyelination does not develop, whereas in mice of identical backgrounds but with *s*, *p*, *v*, *q*, *f* or *r* haplotypes persistent virus infection and demyelinating disease develop.[11] The difference in susceptibility and resistance has been mapped to the D region of the H-2 locus of the major histocompatibility complex (MHC), indicating that a class I-restricted immune response is critical in the process.[12,13] Genetic crosses of congenic mice with different MHC haplotypes on identical B10 backgrounds showed that F_1 progeny derived from resistant and susceptible mice exhibit no or minimal demyelination, indicating that on a B10 background resistance is inherited as a dominant trait.[14] These data are consistent with the hypothesis that the immunologic basis of resistance is determined by efficient presentation of virus antigen to the immune system, resulting in local clearance of virus and subsequent absence of demyelination.

Previous studies demonstrated that immunosuppression with sublethal gamma irradiation renders normally resistant C57BL/10SNJ mice susceptible to TMEV-induced demyelination.[15] These irradiated mice also had increased inflammation surrounding anterior horn cells of the spinal cord, early paralysis, and increased frequency of death, primarily during the third week of infection.[15] In addition, C57BL/10SNJ mice depleted of natural killer (NK) cells by *in vivo* treatment with mAb NK1.1 or polyclonal antiserum anti-asialo-GM1 developed diffused encephalitis, meningitis, and paralysis and died, indicating that NK cells are also critical effectors in protecting against TMEV-induced encephalitis and gray matter disease of the spinal cord.[16] In contrast, treatment of resistant mice with anti-μ IgG in an effort to deplete early B cells has had no effect on pathologic features or clinical variables.[17] Similar results were obtained with certain strains of B-cell-deficient mice that exhibit the *xid* mutations, indicating that immunoglobulins play little role in protecting resistant mice from TMEV infection.[17]

The purpose of the present experiments was to examine the role of T-cell subsets in the immune response to TMEV in resistant mice. C57BL/10SNJ mice were treated with mAbs to CD3, CD4, or CD8 in an effort to deplete or interfere with the function of T-cell subsets to determine more precisely the contribution of these cells to resistance to TMEV disease. Immunocytochemistry to detect virus antigen was used to determine if depletion of T-cell subsets predisposed to virus antigen persistence.

Results

T-cell subset depletion with monoclonal antibody (mAb) GK1.5 (anti-CD4) and mAb 2.43 (anti-CD8)

The efficiency of *in vivo* depletion of $CD4^+$ and $CD8^+$ T lymphocytes was determined by injecting two normal C57BL/10SNJ mice intraperitoneally with a total of 1 mg

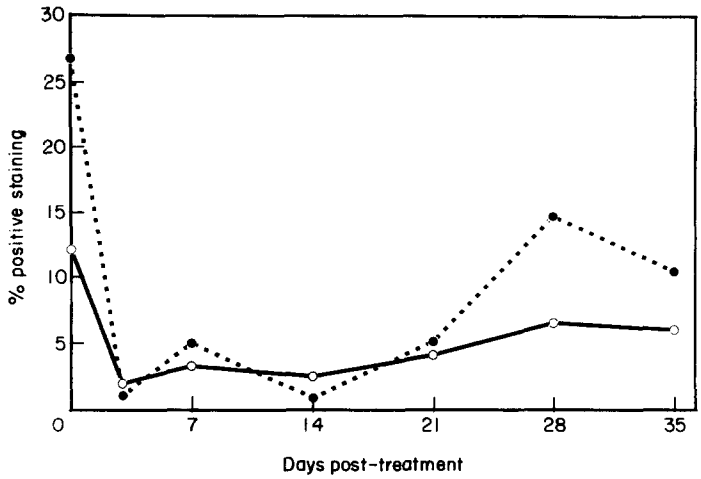

Fig. 1. Percentage of mononuclear cells (by flow cytometry) in blood from C57BL/10SNJ mice given i.p. injections of 1 mg monoclonal GK1.5 (anti-CD4) (···) or 2.43 (anti-CD8) (——) $CD4^+$ T cells (●), $CD8^+$ T cells (○). Values on day 0 represent means of results of five experiments from mice treated with control normal rat IgG (1 mg).

anti-CD4, anti-CD8, or normal rat IgG (NrIgG) over a 2-day period (0.5 mg/day). Blood specimens were drawn weekly and assayed for the presence of $CD4^+$ and $CD8^+$ lymphocytes by flow-cytometric analysis. Mice treated with anti-CD4 or anti-CD8 mAbs demonstrated a substantial decrease in $CD4^+$ and $CD8^+$ T lymphocytes, respectively (Fig. 1). Conversely, treatment with anti-CD4 or anti-CD8 mAbs had minimal effect on the percentage of $CD8^+$ or $CD4^+$ lymphocytes, respectively. $CD4^+$ lymphocytes remained depleted through 21 days post-treatment. By day 35, the percentage of $CD4^+$ lymphocytes increased to 10.4% but still remained at 50% of untreated controls. $CD8^+$ lymphocytes were depleted through 14 days post-treatment but increased to 70% of normal by day 35. Therefore, treatment with anti-CD4 and anti-CD8 mAbs depleted the respective cell types, and levels remained abnormal for the 35 days of the experiment. Previously published experiments demonstrated depletion of both $CD4^+$ and $CD8^+$ T cells after *in vivo* treatment with anti-CD3 mAb.[18] Results of *in vivo* T-cell depletion using mAb GK1.5 or 2.43 in SJL/J mice also have been reported previously.[19]

Agglutination of sheep erythrocytes (SRBCs) in mice treated with mAb to T-cell subsets

To determine the degree of immunosuppression, C57BL/10SNJ mice treated with mAbs to T-cell subsets were injected i.p. with SRBCs (a T-cell-dependent antigen) on day 1, 7, or 28 after virus infection and assays for haemagglutination were done on day 10, 14, or 35 after virus infection, respectively (Table 1). All mice treated with control NrIgG or normal hamster (Nhams) IgG developed an antibody response to SRBCs that resulted in a titer of approximately eight serial doubling dilutions. In contrast, mice treated with anti-CD4 mAb consistently demonstrated undetectable or low titers of agglutination whether the mAb was given at the time of virus infection or 15 days after infection. Similarly, mice treated with anti-CD3 mAb or a combination of anti-CD4 and anti-CD8 mAbs also had undetectable or low agglutination titers. In contrast, mice treated with anti-CD8 mAb showed essentially normal agglutination responses. This documented that partial immunosuppression persisted throughout the

Table 1 Antibody response to sheep erythrocytes in C57BL/10SNJ mice infected with Theiler's murine encephalomyelitis virus and treated with monoclonal antibodies to T-cell subsets

Treatment	Day of SRBC immunization	Day of assay	Mice without titer (%)	Agglutination titer IgM (log_2 mean ± SD)
Anti-CD4[a]	28	35	63.6	2.39 ± 3.54
Anti-CD8[a]	28	35	27.2	6.98 ± 4.57
NrIgG[a]	28	35	0.0	8.60 ± 0.96
Anti-CD4[b]	28	35	100.0	ND
Anti-CD8[b]	28	35	11.1	8.10 ± 3.26
Anti-CD4[a]	7	14	100.0	ND
Anti-CD8[a]	7	14	0.0	8.35 ± 0.0
Anti-CD4 plus anti-CD8[a]	7	14	100.0	ND
Anti-CD3[a]	7	14	66.6	2.45 ± 3.84
NhamsIgG[a]	7	14	0.0	8.86 ± 0.71
Anti-CD4[a]	1	10	100.0	ND

[a] Treatment given at time of virus infection.
[b] Treatment given 15 days after virus infection.
ND, none detected; Nhams, normal hamster; Nr, normal rat; SRBC, sheep erythrocytes.

course of the experiment (35 days after virus infection and mAb treatment) because animals immunized with SRBCs 28 days after anti-CD4 mAb treatment continued to show undetectable or low agglutination titers.

Clinical observations

To determine the role of T-cell subsets in resistance to TMEV infection, C57BL/10SNJ mice were treated i.p. with immunosuppressive doses of mAbs to CD4, CD8 or CD3 either prior to virus infection (days −1 and 0) or during the early phase of disease (days 15, 16 and 17) when demyelination begins to develop in the spinal cord of susceptible mice. Treatment of mice with 400 μg hamster mAb anti-CD3 during early infection resulted in 11 of 13 mice (84.6%) showing either paralysis or death compared to none of five untreated mice (Table 2). None of 11 mice treated with equivalent

Table 2 Clinical data on Theiler's murine encephalomyelitis virus-infected C57BL/10SNJ mice treated with monoclonal antibodies to T-cell subsets

			Paralysis		Death		Total[a]	
Treatment	Day[b]	*n*[c]	No.	Day (mean ± SD)	No.	Day (mean ± SD)	No.	%
Anti-CD4	−1, 0	29	3	13.3 ± 3.1	6	20.8 ± 6.2	9	31.0
Anti-CD8	−1, 0	32	5	8.5 ± 3.0	4	15.0 ± 11.2	9	28.1
Anti-CD3	−1	13	6	11.6 ± 2.6	5	12.6 ± 4.9	11	84.6
Anti-CD4 plus anti-CD8	−1, 0	10	0	0.0 ± 0.0	7	15.2 ± 0.9	7	70.0
None	none	5	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0
NrIgG	−1, 0	21	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0
NhamsIgG	−1	11	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0
Anti-CD4	15, 16, 17	10	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0
Anti-CD8	15, 16, 17	10	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0
Anti-CD3	15	6	1	15	5	23.6 ± 5.8	6	100
NrIgG	15, 16, 17	5	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0
NhamsIgG	15	5	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0

[a] Mice showing paralysis or death prior to scheduled sacrifice on day 35 after virus infection. If an animal showed paralysis and subsequently died, it was counted only in the death column.
[b] Day of antibody treatment (0 to time of virus infection).
[c] Number of mice.
Nhams, normal hamster; Nr, normal rat.

doses of NhamsIgG showed disease (Table 2). After treatment with anti-CD4 or anti-CD8 rat mAbs, 18 of 61 (29.5%) mice developed early paralysis or died. None of 21 mice treated with control NrIgG showed disease. No major difference was noted in death or paralysis between infected C57BL/10SNJ mice treated with anti-CD4 or anti-CD8, with 31 or 28.1% of mice showing disease, respectively. However, paralysis or death occurred sooner in mice treated with anti-CD8 compared to anti-CD4 mAbs (Table 2). To confirm a synergistic role of $CD4^+$ and $CD8^+$ T cells, C57BL/10SNJ mice were treated simultaneously with anti-CD8 and anti-CD4 mAbs. Seven of 10 mice died at approximately 15 days after infection. All of these mice were paralysed prior to death. The experiment was scheduled for termination on day 35 after TMEV infection, so it was not possible to determine if these treatments would have resulted in delayed paralysis or death.

A different effect was observed when infected C57BL/10SNJ mice were treated with mAbs beginning 15 days after infection. Death occurred approximately 3 weeks after infection in all six mice treated with anti-CD3 mAb. However, no paralysis or death was observed when anti-CD4 or anti-CD8 mAbs were given independently. This indicated that at this phase of disease either of the T-cell subsets could mediate resistance to disease but depletion of both subsets resulted in death. No paralysis or death was observed in C57BL/10SNJ mice treated with control antisera after 15 days of infection.

Pathologic observations (early disease)

A detailed pathologic analysis was performed in spinal cord and brain of infected C57BL/10SNJ mice that were treated with mAbs and survived until scheduled termination of the experiment. On day 10 after infection, an increased degree of anterior horn cell necrosis, inflammation and neuronal inflammation in the brain were observed in mice treated with anti-CD8 or anti-CD3 mAbs compared to mice receiving NrIgG (Table 3). The major pathologic difference detected was in the degree of necrosis and inflammation in the pyramidal layer of the hippocampus and in the thalamus (Fig. 2). In control mice treated with NrIgG, the majority of brains showed mild inflammatory changes in hippocampus. In contrast, mice immunosuppressed by treatment with anti-CD3, anti-CD8, and, to a lesser extent, anti-CD4 mAb showed prominent necrosis in the hippocampus and deep within the thalamus. However, by

Table 3 Treatment of resistant C57BL/10SNJ mice with monoclonal antibodies to T-cell subsets (early pathologic changes)

Treatment	n^a	Day of termination	Spinal cord score: Gray matter inflammation	Spinal cord score: Meningeal inflammation	Spinal cord score: Demyelination	Total brain score
Anti-CD4[b]	6	10	1.0±1.1	0.0±0.0	0.0±0.0	1.5±1.7
Anti-CD8[b]	8	10	5.8±7.4	0.7±1.4	0.2±0.6	5.4±3.6
Anti-CD3[c]	9	10	4.2±5.2	1.0±1.6	1.1±1.9	5.3±3.3
Anti-CD4 plus anti-CD8[b]	4	10	2.6±5.2	0.0±0.0	0.0±0.0	3.2±2.5
NrIgG[b]	8	10	1.8±1.8	0.0±0.0	0.0±0.0	2.1±1.6
Anti-CD4[b]	7	14	0.2±0.6	0.0±0.0	0.0±0.0	6.4±1.8
Anti-CD8[b]	6	14	0.9±1.6	1.6±2.5	0.3±0.7	2.7±1.6
NrIgG[b]	6	14	0.9±1.7	0.0±0.0	0.3±0.8	2.2±2.3

[a] Number of mice.

[b,c] Mice infected with Theiler's murine encephalomyelitis virus (day 0) were treated i.p. with purified antibody on days −1 and 0[b] or day −1[c].

Nr, normal rat.

Values represent mean ± SD.

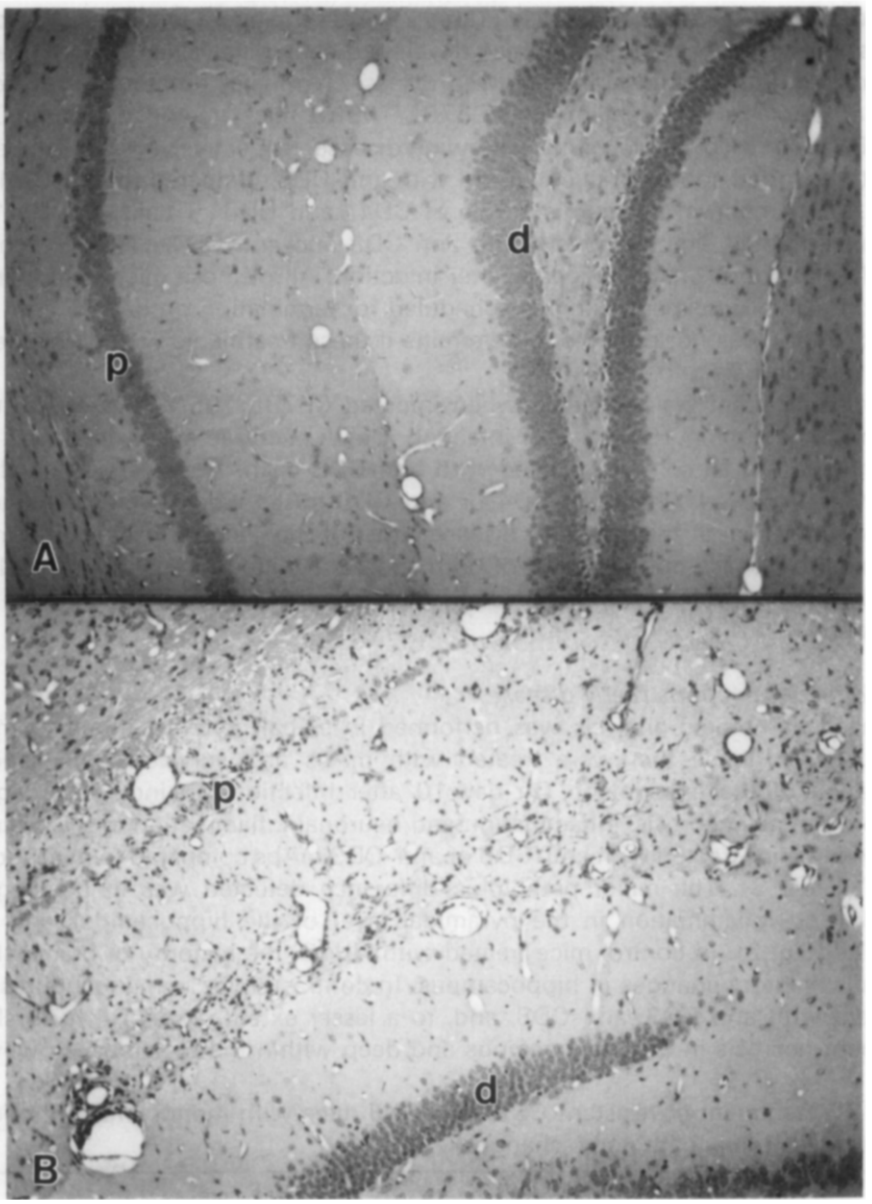

Fig. 2. Sections of hippocampus from Theiler's murine encephalomyelitis virus-infected C57BL/10SNJ mice (7 days) treated with normal rat IgG (A) or monoclonal antibody (mAb) anti-CD4 (B). Note extensive neuronal necrosis and inflammatory infiltrates in pyramidal (p) layer of hippocampus from mouse treated with mAb anti-CD4 (B) but not in control animal (A). Inflammatory infiltrates are also present in stratum radiatum and in molecular layer of hippocampus but not in dentate (d). (Paraffin-embedded section stained with hematoxylin–eosin; ×140.)

day 14 after infection, this difference between treatment groups was less apparent. No increase in the degree of meningeal inflammation or spinal cord demyelination was observed in resistant mice depleted of T-cell subsets during the early phase of disease (day 10 or 14 after infection). No pathologic abnormalities were noted in the cerebellum in any of the mice, irrespective of treatment.

Pathologic observations (late disease)

To determine if early depletion of T-cell subsets would allow for the subsequent

Table 4 Treatment of resistant C57BL/10SNJ mice with monoclonal antibodies to T-cell subsets (late pathologic changes)

Treatment	n^a	Day of termination	Spinal cord score: Gray matter inflammation	Spinal cord score: Meningeal inflammation	Spinal cord score: Demyelination	Total brain score
Anti-CD4[b]	14	35	5.0±11.5	4.3±6.2	5.4±7.3[c]	1.4±1.2
Anti-CD8[b]	20	35	1.5±2.9	2.9±4.5	3.9±3.7[d]	1.2±1.0
Anti-CD3[e]	3	35	0.6±1.0	1.2±2.1	0.6±1.0	—
NrIgG[b]	14	35	0.4±1.2	0.3±1.0	2.1±4.9[f]	1.3±1.5
None	5	35	0.0±0.0	0.4±0.8	0.4±0.8	—

[a] Number of mice.
[c] $P = 0.023$.
[d] $P = 0.0006$.
[f] $P = 0.234$ (not significant).
c, *d* and *e* are compared to demyelination scores of infected mice receiving no treatment (Student's *t*-test assuming unequal variance).
Mice infected with Theiler's murine encephalomyelitis virus (day 0) were treated i.p. with total of 1.0 mg purified antibody in divided doses on days −1 and 0[b] or day −1[e].
—, Not done; Nr, normal rat.
Values represent mean±SD.

development of CNS demyelination, spinal cords from C57BL/10SNJ mice were analysed on day 35 after infection (Table 4) using plastic-embedded sections. More demyelinating lesions were observed in mice treated with mAbs to CD4 or CD8 than in controls (Fig. 3), but this difference was minor and did not equal the degree of demyelination and inflammation observed normally in susceptible strains of mice.[19] The results with anti-CD3 mAb treatment were inconclusive because the majority of these mice died prior to scheduled sacrifice on day 35. However, earlier analysis of these mice on day 10 (Table 3) failed to demonstrate an increase in demyelination.

To determine if late immunosuppression would result in demyelination, C57BL/10SNJ mice were treated with mAbs beginning on day 15 after infection and analysed for pathologic abnormalities on day 35 (Table 5). No increase in demyelination or inflammation was seen in the spinal cord of these mice. Again, the results with anti-CD3 mAb were inconclusive because only one of six mice survived; this mouse showed a small number of demyelinated lesions in the spinal cord but did show evidence of encephalitis. Of interest, treatment with anti-CD4, anti-CD8, or anti-CD3 mAb beginning on day 15 after infection resulted in an increased total brain score, reflecting neuronal necrosis and inflammation in the deep structures of the brain.

Virus antigen production in C57BL/10SNJ mice treated with mAbs to T-cell subsets

Immunocytochemical[7] and *in situ* hybridization studies[20] indicated a strong correlation between local virus replication and area of pathologic change. In C57BL/10SNJ mice, lack of pathologic abnormalities strongly correlates with efficient virus clearance.[14] To determine if depletion of T-cell subsets would predispose to virus antigen persistence on day 35 after infection, serial spinal cord sections from mice previously perfused with Trump's fixative were stained for virus antigen. Spinal cord sections from virus-infected mice treated with control NrIgG did not have virus-antigen-positive cells. In contrast, all mice studied that were treated with anti-CD3, anti-CD8, or anti-CD4 mAbs showed virus-antigen-positive cells in the spinal cord white matter (Fig. 4). Many of the virus-antigen-positive cells were associated with small areas of demyelination and with a modest cellular infiltrate consisting primarily of macrophages.

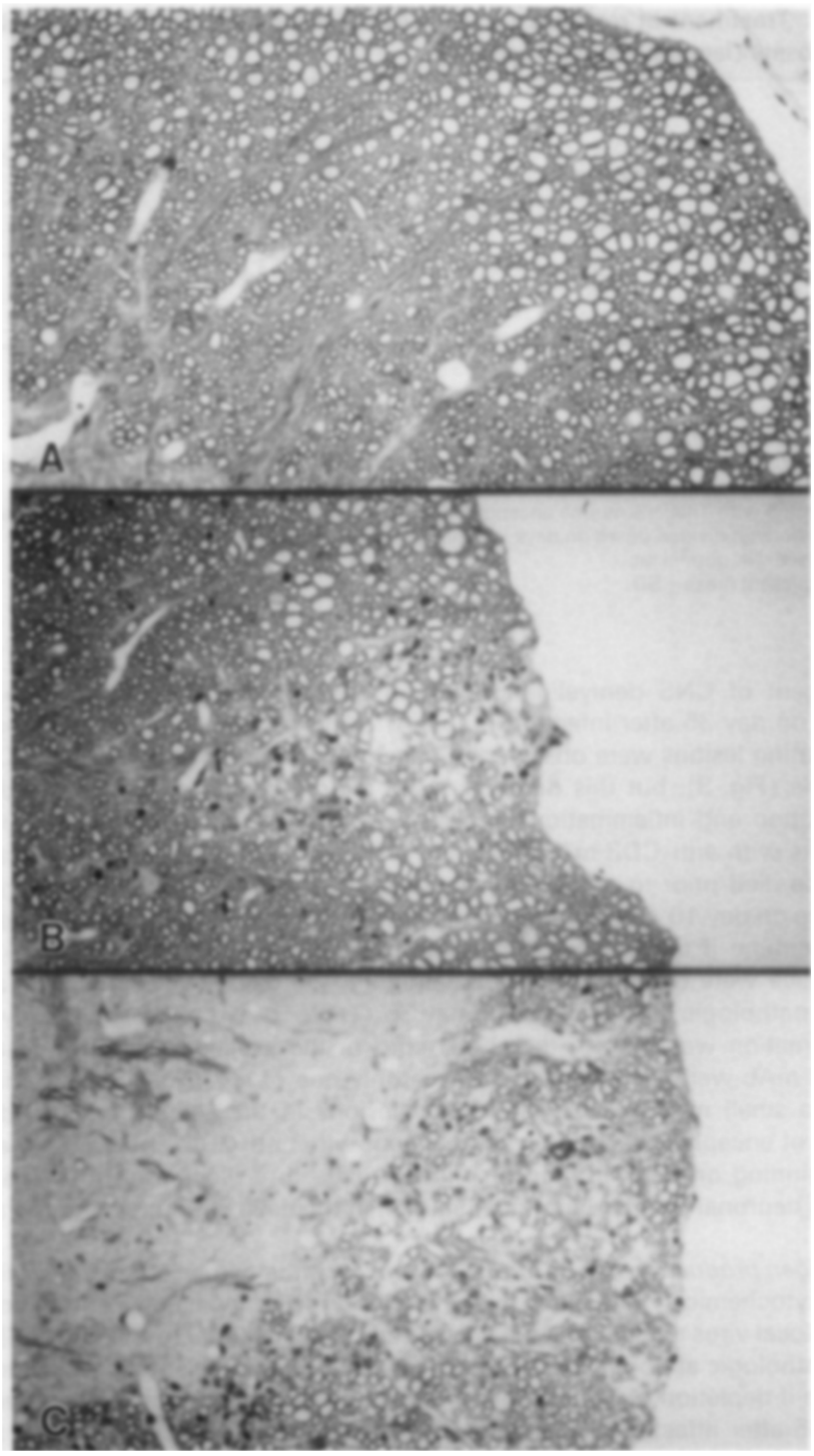

Fig. 3. Sections of spinal cord (35 days) from Theiler's murine encephalomyelitis virus-infected C57BL/10SNJ mice treated with normal rat IgG (A), monoclonal antibody (mAb) GK1.5 (anti-CD4) (B), or mAb 2.43 (anti-CD8) (C). Focal areas of primary demyelination (and relative absence of inflammatory infiltrates) are present in spinal cords of mice depleted of T-cell subsets (B and C). Similar lesions were observed in mice depleted of all T cells by treatment with anti-CD3 mAb or combined treatment with anti-CD4 and anti-CD8 mAbs. (Glycol methacrylate-embedded section stained with modified erichrome–cresyl violet stain; ×870.)

Table 5 Treatment of resistant C57BL/10SNJ mice with monoclonal antibodies to T-cell subsets (late pathologic changes after treatment beginning on day 15)

Treatment	n^a	Day of termination	Spinal cord score: Gray matter inflammation	Spinal cord score: Meningeal inflammation	Spinal cord score: Demyelination	Total brain score
Anti-CD4[b]	10	35	0.0±0.0	0.0±0.0	0.0±0.0	2.9±2.3
Anti-CD8[b]	9	35	0.1±0.4	0.4±1.3	1.9±3.1	4.0±3.8
Anti-CD3[c]	1	35	0.0	2.3	2.3	7.0
NrIgG[b]	5	35	0.0±0.0	0.0±0.0	0.4±0.8	0.4±0.9
NhamsIgG[c]	4	35	0.0±0.0	0.3±0.7	0.3±0.7	0.0±0.0

[a] Number of mice.

[b,c] Mice infected with Theiler's murine encephalomyelitis virus on day 0 were treated with 1 to 3 mg purified antibody in divided doses on days 15, 16 and 17[b] or day 15[c].

Nhams, normal hamster; Nr, normal rat.

Values represent mean±SD.

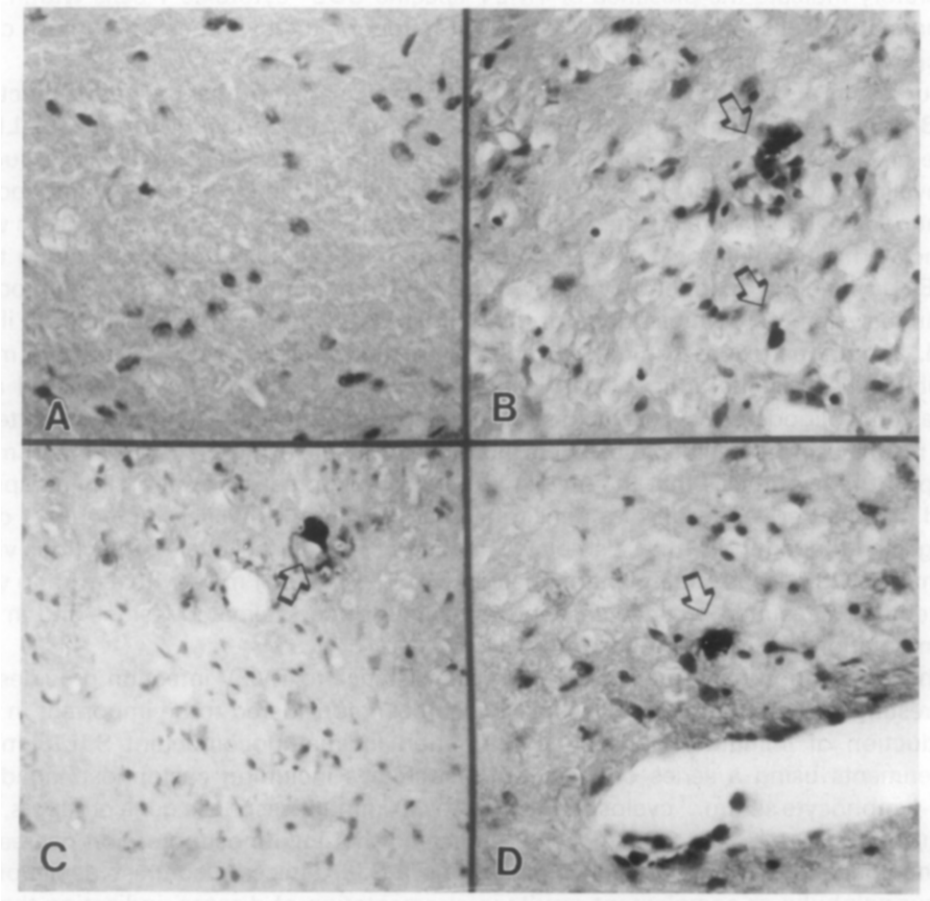

Fig. 4. Detection of virus antigen in spinal cord of mice infected with Theiler's murine encephalomyelitis virus (TMEV) (35 days) and treated with normal rat IgG (A), anti-CD8 monoclonal antibody (mAb) (B), anti-CD4 mAb (C), or anti-CD3 mAb (D). Numerous virus antigen-positive glial cells (arrows) were detected in spinal cord white matter in mice depleted of T cells (B, C and D) but none were detected in non-immunosuppressed C57BL/10SNJ TMEV-infected mice. (Immunoperoxidase staining using rabbit anti-TMEV polyclonal antisera on frozen sections counterstained with haematoxylin to detect demyelination and inflammation; ×870.)

Discussion

These experiments demonstrated that both $CD4^+$ and $CD8^+$ T cells play a role in the resistance to Theiler's virus-induced infection. Depletion of $CD4^+$ or $CD8^+$ T cells in infected C57BL/10SNJ mice resulted in increased frequency of paralysis and early death secondary to neuropathologic injury to the pyramidal neurons of the hippocampus, thalamus, and anterior horn cells of the spinal cord gray matter. $CD4^+$ and $CD8^+$ T cells appear to work in a synergistic fashion because depletion or inhibition of both the subsets by treatment with anti-CD3 mAb or by combined treatment with anti-CD4 and anti-CD8 mAbs resulted in augmented neurovirulence of the virus with subsequent paralysis of mice and death. Depletion of either T-cell subset resulted in virus antigen persistence in the spinal cord, indicating that both $CD4^+$ and $CD8^+$ T cells are required for the clearance of virus from the CNS. These results are similar to those reported by Williamson and Stohlman[21] for the mouse hepatitis virus model system. Infection with this coronavirus also induces demyelination in the spinal cord. These authors concluded that clearance of mouse hepatitis virus is mediated by virus-specific $CD8^+$ T cells with the help of $CD4^+$ T cells. *In vitro* experiments from our laboratory indicate the presence of TMEV-specific $CD8^+$ cytotoxic T cells within the inflammatory cells infiltrating the CNS.[22] This supports the hypothesis that these cells along with $CD4^+$ T cells play a role in limiting virus infection *in vivo*.

Our results are also consistent with the observation made by others[23–25] after infection of BALB/c mice homozygous with respect to the nude gene (*nu/nu*). BALB/c mice are similar to C57BL/10SNJ mice in that they are resistant to TMEV-induced demyelination. However, when BALB/c (*nu/nu*) mice, which have a T-lymphocyte immunodeficiency, were injected with TMEV, they developed paralysis, and death was associated with small focal areas of demyelination in the spinal cord, indicating that TMEV is lytic to oligodendrocytes without the contribution of the T-lymphocyte immune system. In contrast, none of the normal BALB/c mice became clinically ill or died. Immunoperoxidase studies demonstrated that infected BALB/c (*nu/nu*) mice exhibited heavy virus-antigen load in the anterior horn cells of the spinal cord and occasional oligodendrocytes expressed virus antigen in the spinal cord white matter.[24] Our results using T-cell depleted mice and those of others using BALB/c (*nu/nu*) mice support the hypothesis that the inability to clear virus infection from brain and spinal cord gray matter is a result of a deficit in T-cell function. However, more recent data suggested that antibody may also play a role in decreasing the level of infectious virus from the CNS because treatment of infected BALB/c (*nu/nu*) mice with a mAb to viral protein VP-1 resulted in a decrease in the number of demyelinated lesions and in the augmentation of CNS remyelination.[25]

The observation that T cells play a role in resistance to TMEV infection provides an interesting paradox because T cells have also been considered to be important in the production of immune-mediated demyelination in immunocompetent SJL/J mice. Experiments using a series of therapeutic strategies including cyclophosphamide,[26] anti-lymphocyte serum,[27] cyclosporine,[28] mAbs to immune response gene products,[29,30] or mAbs to $CD4^+$[19,31] or $CD8^+$ T cells[19] indicated that immunosuppression decreases the number and extent of demyelinating lesions in SJL/J mice. In contrast, depletion of immunoglobulin or complement results in augmentation of disease, indicating that in this case antibodies may be protective.[32] More recent experiments from our laboratory indicate that *in vivo* treatment with tumour necrosis factor alpha results in a marked reduction of demyelinated lesions in susceptible SJL/J mice.[33]

To resolve this paradox we propose that resistant mice develop an early protective immune response consisting both of NK cells and T cells directed against virus antigens

that clears virus from the brain and spinal cord gray matter. Because of a deficit in NK function[16] or antigen presentation, susceptible SJL/J mice are unable to clear virus. This allows for subsequent infection of oligodendrocytes, astrocytes, and macrophages in the spinal cord white matter that may be lysed during chronic infection by previously primed cytotoxic T lymphocytes directed against virus antigens or virus-induced host antigens. The persistence of TMEV in the presence of antiviral cytotoxic T lymphocytes may be the result of virus mutants that arise during infection that can evade antiviral cytotoxic lymphocytes.

Methods

Virus. The Daniels (DA) strain of TMEV was used for all experiments.[34] This strain, originally obtained from J. R. Lehrich and associates of the University of Chicago,[35] was grown to 5×10^8 plaque-forming units/ml in baby hamster kidney-21 cells.

Antibodies. Cells of hybridomas GK1.5 and 2.43, both of which synthesize rat antibody of the IgG2b isotype, were obtained from the American Type Culture Collection. mAb GK1.5 and 2.43 recognize monomorphic determinants on L3T4 (CD4) and Lyt-2.2 (CD8) molecules, respectively. Hamster mAb 145-2C11 directed against mouse CD3[18,36,37] was obtained from J. A. Bluestone (University of Chicago). mAbs obtained from ascites fluid were used for *in vivo* therapy and for staining cells in flow cytometry. To prepare ascites fluid, 1×10^7 hybridoma cells were injected into BALB/c mice that had been primed with 2,6,10,14-tetramethylpentadecane and irradiated sublethally with 5 Gy or into T-cell-deficient BALB/c nude mice. Ascites fluid containing the mAb was purified by affinity chromatography on a mouse anti-rat IgG column (anti-CD4 or CD8 mAbs) or an anti-hamster IgG column (anti-CD3 mAb).

Flow cytometry. Two hundred microliters of a suspension of washed cells from peripheral blood (5×10^6/ml) was incubated on ice for 20 min with an equal volume of a 1/500 dilution of purified ascites fluid (1–2 mg/ml) containing anti-Lyt-2 (CD8) or anti-L3T4 (CD4) mAb. After washing, the cells were stained with biotin-conjugated mouse anti-rat IgG (Vector Laboratories, Burlingame, California) that did not cross-react with mouse Ig. After incubation on ice for another 30 min, cells were washed twice, stained 15 min with 0.1 ml of streptavidin-fluorescein isothiocyanate, conjugated, diluted 1:500, and analysed by flow cytometry (FACS V; Becton Dickinson, San Jose, California). To evaluate the degree of background staining, lymphocytes were stained by substituting the primary antibody with NrIgG.

Animals and experimental protocol. C57BL/10SNJ (H-2^b) female mice (4 to 6 weeks old) from The Jackson Laboratory (Bar Harbor, Maine) were innoculated intracerebrally with 2×10^5 plaque-forming units of TMEV in 10-μl vehicle. Mice were treated i.p. with 0.4 mg anti-CD3, 1 or 2 mg anti-CD4 or CD8, 1 mg NrIgG or NhamsIgG, either at the time of virus inoculation or 15 days after infection. Animals were monitored daily for presence of paralysis or death until day 7, 10, 14, or 35 of scheduled termination of the experiment.

Pathologic Analysis. Mice were perfused with Trump's fixative (phosphate-buffered 4% formaldehyde containing 1% glutaraldehyde, pH 7.4) and the spinal cords were processed to provide 2 μm thick glycol methacrylate-embedded sections that were stained with a modified erichrome stain. A detailed morphologic analysis was performed on each of 10 to 15 coronal spinal cord sections from each animal. This resulted in the analysis of 150 to 300 spinal cord sections in each experimental group. A pathologic score based on gray matter inflammation, meningeal inflammation, and demyelination was obtained for each animal as described previously.[19] Each quadrant of every spinal cord section was graded for the presence or absence of pathologic features. The maximum pathologic score, 100, indicated the presence of inflammation or demyelination or both in every quadrant of all spinal cord sections of one mouse. All data are presented as mean $\pm$ standard deviation.

Brains from perfused animals were removed from the skull, cut into three coronal sections, and embedded in paraffin. The cerebellum, brain stem, hippocampus, striatum, cerebral cortex, meninges, and corpus callosum were graded independently on a four-point scale for the presence of inflammation and necrosis (0, no pathologic abnormalities; 1, minimal inflammation

with <25 cells per 40× high-power field (HPF); 2, moderate inflammation with 25 to 50 inflammatory cells per HPF; 3, intense inflammation with >50 inflammatory cells per HPF and early necrosis; and 4, intense inflammation and widespread necrosis of tissue). The scores from the various areas were added to provide a total brain score (maximum = 28).

Sheep erythrocyte agglutination assay. Serum samples from treated mice were assayed for IgM hemagglutination of SRBCs to determine whether i.p. treatment with mAbs to T-cell subsets suppressed the humoral immune response to an irrelevant antigen. Animals were given 1×10^9 SRBCs by i.p. injection 7 days before sacrifice or assay. Sera from these mice were stored at −70°C and assayed at one time for IgM hemagglutination. Multiple serial dilutions of the sera (1:10 through 1:10240) were made, and 100 μl of each dilution was placed on 96-well microtiter plates. A 1:3000 dilution of packed SRBCs (in phosphate-buffered saline-0.1% albumin) was made, and 50 μl was added to each well. This mixture was incubated at 37°C for 30 min and then spun for 2 min at 2000 rpm. The agglutination was read to determine the IgM titer.

Immunocytochemistry to detect virus antigen. For immunoperoxidase studies, spinal cord tissue blocks stored in 0.1 M phosphate buffer were rinsed in 0.1 M Tris buffer with 25 mM hydroxylamine (pH 7.4), treated with 10% dimethyl sulphoxide in the same buffer for 1 h, and quick-frozen in isopentane chilled in liquid nitrogen. Ten-micrometer cryostat sections were cut and transferred to gelatin-coated glass slides. Frozen sections of spinal cords or deparaffinized brain sections from mice perfused with Trump's fixative were reduced with 1% sodium borohydride in 0.1 M Tris buffer with 25 mM hydroxylamine (pH 7.4) at 4°C and refixed with 95% alcohol–5% glacial acetic acid. Sections were immunostained with a polyclonal antiserum to purified TMEV strain DA virions[7] by the avidin–biotin immunoperoxidase technique (Vector Laboratories, Burlingame, California). Slides were developed with a solution of Hanker-Yates reagent (p-phenylenediamine-procatechol [Polysciences, Warrington, Pennsylvania]) and counterstained with hematoxylin.

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