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Coronavirus-Induced Demyelination Occurs in the Presence of Virus-Specific Cytotoxic T Cells

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C57Bl/6, but not BALB/c, mice infected with mouse hepatitis virus strain JHM (MHV–JHM) develop a late onset, symptomatic demyelinating encephalomyelitis. In this report, we characterized anti-viral cytotoxic T cells in the central nervous system and spleen during the acute and chronic stages of the MHV infection. The data show that C57Bl/6 mice display a cytotoxic T cell (CTL) response to the surface (S) glycoprotein and this response can be demonstrated in lymphocytes isolated from the brains and spinal cords of mice both acutely and persistently infected with MHV–JHM. Thus, the anti-S CTL activity present in the central nervous system of chronically infected animals is not sufficient to prevent the demyelinating process. BALB/c mice have been shown previously to mount a CTL response against the nucleocapsid (N) protein (Stohlman *et al.*, 1992). Since C57Bl/6 mice do not mount a response to the N protein, the role of the N-specific response in preventing the late onset disease was assessed using B10.A(18R) mice, recombinant in the H-2 locus. These mice contain the d alleles of the D and L loci and exhibit a CTL response against the N protein. However, unlike the BALB/c mice, these animals develop the late onset symptomatic disease. These results suggest that the N-specific response is partially protective against the development of the demyelinating disease, but that additional factors are also likely to be involved.

INTRODUCTION

Coronaviruses, single-stranded, positive polarity RNA viruses, cause a variety of acute and chronic infections in many species of animals (Siddell *et al.*, 1983). Mouse hepatitis virus, strain JHM (MHV–JHM), is neurotropic and causes acute encephalitis and acute and chronic demyelinating encephalomyelitis in susceptible mice and rats (Cheever *et al.*, 1949; Lampert *et al.*, 1973; Weiner, 1973; Nagashima *et al.*, 1978; Sorensen *et al.*, 1980; Perlman *et al.*, 1987a). Most strains of mice invariably develop an acute fatal disease after intracerebral or intranasal inoculation with MHV–JHM. A fatal outcome can be prevented if mice are treated with a passive infusion of antibody or T cells from immunized animals or if they are infected with an attenuated strain of virus (Haspel *et al.*, 1978; Buchmeier *et al.*, 1984; Dalziel *et al.*, 1986; Nakanaga *et al.*, 1986; Stohlman *et al.*, 1986; Lecomte *et al.*, 1987; Fleming *et al.*, 1989; Körner *et al.*, 1991; Yamaguchi *et al.*, 1991).

Suckling C57Bl/6 mice inoculated intranasally with MHV–JHM at 10 days of age and nursed by immunized dams are fully protected against acute encephalitis. However, 40–90% develop a late onset, demyelinating encephalomyelitis at 3–8 weeks postinoculation (p.i.) (Perlman *et al.*, 1987a). This disease is characterized clinically by hindlimb paralysis and infectious virus can be isolated from these animals. This virus cannot be distinguished by clinical or biochemical criteria from the virus used for the initial infection (Perlman *et al.*, 1990b), showing that in this model, development of MHV variants during the *in vivo* infection is not the explanation for the ability of the virus to persist. In contrast, suckling BALB/c mice inoculated with virus and nursed by immunized dams do not develop a late onset clinical disease (Perlman *et al.*, 1987b), although BALB/c mice are at least as susceptible to the acute infection caused by MHV–JHM as the C57Bl/6 mice (Stohlman and Frelinger, 1978).

The immune response to MHV–JHM has been partially characterized in BALB/c and C57Bl/6 mice. Neutralizing antibodies, whether administered passively or developed during the course of the infection, do not protect mice from the development of the chronic infection (Stohlman and Weiner, 1981; Buchmeier *et al.*, 1984; Watanabe *et al.*, 1987; Jacobsen and Perlman, 1990). Rather, the cell-mediated response appears to be essential for virus clearance and prevention of the persistent infection. Both CD4 and CD8 cells are required for virus clearance from the brains of infected mice (Sussman *et al.*, 1989; Williamson and Stohlman, 1990). CD4 cells which proliferate in response to the surface (S) and transmembrane (M) proteins have been identified in C57Bl/6 mice (Mobley *et al.*, 1992) and CD8 L^d-restricted cytotoxic cells responsive to the nu-

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cleocapsid (N) protein have been described in BALB/c mice (Stohlman *et al.*, 1992, 1993). This CD8 response was detected in the brains of mice with encephalitis and in the spleen after intraperitoneal inoculation. We and others (S. Stohlman, personal communication) have not been able to detect a cytotoxic T cell (CTL) response against the N protein in C57Bl/6 mice which were immunized against or infected with MHV–JHM. In Lewis rats, a CD4 response can be detected against both the N and S proteins, whereas only the S protein appears to be a target for CD8 cytotoxic activity. Both virus-specific CD4 and CD8 cells are able to prevent the acute encephalitis and clear virus in MHV-infected rodents (Körner *et al.*, 1991; Yamaguchi *et al.*, 1991; Flory *et al.*, 1993).

In this communication, we show that CD8 cells with activity against the S protein can be isolated from the brains and spinal cords of C57Bl/6 mice acutely or persistently infected with MHV–JHM, demonstrating that the presence of these cells does not protect mice against the chronic demyelination. We also show, using congenic mice recombinant in the H-2 gene, that a CD8 CTL response against the N protein is not protective against the development of the symptomatic, chronic disease.

MATERIALS AND METHODS

Viruses

MHV–JHM, originally obtained from Dr. S. Weiss, University of Pennsylvania, was grown and titered as previously described (Perlman *et al.*, 1987a). Vaccinia virus, strain WR, was obtained from the ATCC. Recombinant vaccinia virus containing the M and N genes downstream of a T7 promoter were constructed as previously described (Mobley *et al.*, 1992). A recombinant virus containing an S gene lacking the transmembrane domain, and thereby incapable of insertion into the cell membrane, was obtained from Dr. M. Buchmeier, The Scripps Research Institute, and Dr. T. Gallagher, Loyola University School of Medicine. This construct was prepared by sequential treatment with the restriction enzyme *Nde*I and Klenow, resulting in an inframe termination codon at nucleotide 3927. This truncated S gene was also located downstream from the T7 promoter.

Cells

J774.1 (H-2^d) cells were obtained from Dr. S. Stohlman, University of Southern California, and grown in DMEM supplemented with 10% fetal calf serum and antibiotics. MC57 (H-2^b) cells, obtained from Dr. M. Buchmeier, The Scripps Research Institute, were grown in RPMI supplemented with 10% fetal calf serum and antibiotics.

Animals

MHV-negative 6-week-old BALB/c and C57Bl/6 mice were purchased from Sasco Laboratories (Omaha, NE). The B10.A(18R) ($K^bD^dL^d$) mice were obtained from Dr. M. Buchmeier, The Scripps Research Institute. To obtain mice with acute encephalitis, animals were intranasally inoculated with 5×10^4 PFU of MHV–JHM and harvested 6–7 days p.i. To obtain persistently infected mice with hindlimb paralysis, suckling mice were inoculated intranasally with live MHV–JHM and nursed by dams with high titers of anti-MHV–JHM antibody as previously described (Perlman *et al.*, 1987a). Neutralization titers were determined using a constant virus-variable serum plaque reduction assay as described previously (Perlman *et al.*, 1987a).

In situ hybridization

Sequential sections from infected mouse brains were analyzed for viral RNA by *in situ* hybridization and for CD4 and CD8 cells by immunohistochemistry. *In situ* hybridization was performed as previously described using an ^{35}S-labeled RNA probe (Perlman *et al.*, 1990a). The RNA probe was complementary to MHV genes 5 and 6 and to 200 bases of genes 4 and 7. Slides were dipped in NTB-2 nuclear emulsion prior to exposure for 2 weeks at 4°. For each experiment, a negative (uninfected brain) and a positive (mouse dying from acute encephalitis) control were analyzed in parallel. No annealing was detected by film autoradiography when uninfected brains were incubated with the MHV probe. Positive signals were detected on several sequential sections, confirming the specificity of the reaction.

Detection of CD4 and CD8 by immunocytochemistry

Brains and spinal cords were assayed for CD4 and CD8 cells by a modification of a previously described method (Lo *et al.*, 1992). Ten-micrometer-thick frozen sections were placed onto silane-treated slides, dried for 1 hr and fixed for not more than 30 sec in 1% paraformaldehyde. Longer periods of fixation diminished detection of CD4 and CD8 antigen on the cell surface. Sections were outlined with a hydrophobic ring, using a Pap Pen (Research Products International, Mt. Prospect, IL). After washing in phosphate-buffered saline (PBS), sections were placed in primary rat monoclonal antibodies [5 μg/ml anti-L3T4 or 20 μg/ml anti-Ly-2 and 10 μg/ml anti-Ly-3 (PharMingen, San Diego, CA)] for 1 hr at room temperature. After washing, sections were placed in biotin-conjugated goat anti-rat antibody (2.5 μg/ml) (Southern Biotech, Birmingham, AL) for 30 min and then in streptavidin-conjugated horseradish peroxidase (5 μg/ml) (Jackson Immunoresearch Laboratories, West Grove, PA) for an additional hour. Peroxi-

dase was detected with 3-amino-9-ethylcarbazole (AEC) (Sigma Immunochemicals, St. Louis, MO) and hydrogen peroxide. Sections were either not counterstained or lightly counterstained with hematoxylin. Preliminary experiments were performed using diaminobenzidine, with equivalent results. Four mice with acute encephalitis and nine mice with chronic encephalomyelitis were analyzed in these immunocytochemistry experiments, with similar results in all cases.

Isolation of lymphocytes from spleens, brains, and spinal cords

Cells were isolated from the spleens of infected animals as previously described (Mobley *et al.*, 1992). A modification of a previously described procedure was used to isolate cells from the central nervous system (CNS) (Lindsley and Rodriguez, 1989). Briefly, mice were perfused with phosphate-buffered saline and brains and spinal cords removed. Tissue was ground between frosted glass slides and titurated by vigorous pipetting in 5 ml RPMI medium with 10% fetal calf serum. Following thorough dispersion of the tissue, Percoll (Pharmacia, Uppsala, Sweden) was added to a final concentration of 30%. The lysate was spun at 1300 *g* for 30 min at 4°. The Percoll and lipid layers were aspirated and the cell pellet was washed twice with RPMI medium, counted, and prepared for fluorescence-activated cell sorter (FACS) analysis. We were able to isolate substantial numbers of cells from the brains and spinal cords of acutely infected mice at 6–7 days p.i. (mean 2.7×10^6 cells/brain and spinal cord, $n = 45$) and from those of chronically infected mice (mean 1.8×10^6, $n = 15$). Using this method, approximately 10^5 cells were isolated from the brains of uninfected mice or of mice at early times p.i.

FACS analysis

For analysis of cells infiltrating the brain, cells isolated from Percoll gradients were washed in Hanks' buffered saline solution (HBSS) with 10% horse serum, resuspended to a concentration of 2×10^6/ml in HBSS containing 50% (v/v) heat-inactivated normal rat serum, and incubated for 30 min on ice to block Fc receptor binding. One hundred microliters (2×10^5 cells) were then stained for flow cytometric analysis as previously described using the following monoclonal antibodies directly conjugated to phycoerythrin (PE) or cyanine: 53-6.72, anti-CD8; GK1.5, anti-CD4; RA3-6B2, anti-B220 (Mobley and Dailey, 1992). Stained cells were analyzed on a dual laser FACS 440 (Becton–Dickinson, Mountain View, CA). Fluorescence, forward light scatter, and orthogonal light scatter signals were collected on 10,000 cells and analyzed using the FACS/DESK computer program on a VAX 3200. Forward angle light scatter and orthogonal light scatter were used to exclude dead cells from analysis.

Primary CTL assays

J774.1 or MC57 cells dually infected with recombinant vaccinia virus (VV) expressing MHV genes and T7 RNA polymerase, each at an m.o.i. of 3, were used as targets. Following a 6-hr incubation at 37°, cells were washed and 1×10^6 cells were labeled with 0.1 mCi $Na^{51}CrO_4$ (Amersham, Arlington Heights, IL) in 1 ml for 2 hr at 37°. Cells were then centrifuged through an equal volume of bovine serum, resuspended in RPMI with 10% bovine serum, and allowed to sit for 15–30 min at room temperature. Cells were washed, counted, and added to the effector cells in 96-well round-bottom microtiter dishes (Costar, Cambridge, MA). Each effector–target combination was analyzed in triplicate. Cells isolated from the brains of 2–4 mice were pooled in each experiment, to obtain a sufficient number of effectors.

After 4 hr incubation at 37°, the cells were spun at 1000 *g* for 5 min at room temperature and 0.1 ml of supernatant from each reaction was placed in a scintillation vial. Chromium release was measured in a liquid scintillation counter (Beckman Instruments, Fullerton, CA). Spontaneous release was the amount of radioactivity released in the absence of effector cells. The average spontaneous release was 25.0% (SD 4.5%) for the J774.1 cells and 22.6% (SD 5.8%) for the MC57 cells. CTL activity was calculated using the formula

$$\% \text{ Specific lysis} = \frac{(\text{experimental release}) - (\text{spontaneous release})}{(\text{maximum release [NP-40-treated]}) - (\text{spontaneous release})} \times 100$$

Complement lysis

Cells isolated from the brain and spinal cord or the spleen were treated with a mAB specific for CD8 cells (HO-2.2) (Raulet *et al.*, 1980) or for CD4 cells (GK1.5). After washing with HBSS medium supplemented with 10% bovine serum, cells were resuspended in RPMI medium supplemented with 10% fetal calf serum. Cells were then treated with complement (Pel-Freez, Brown Deer, WI) for 40 min at 37° and subsequently washed. The efficacy of the treatment was determined to be >90% by FACS analysis. The remaining cells were used in a CTL assay as described above.

RESULTS

Isolation of lymphocytes from the CNS of acutely and chronically infected C57Bl/6 mice

C57Bl/6 mice inoculated intranasally with MHV–JHM develop a uniformly lethal acute encephalomyeli-

TABLE 1

PHENOTYPE OF CELLS INFILTRATING THE CNS OF INFECTED MICE

Disease	No. of mice	CD4[a]	CD8[a]	CD4/CD8	B[a]
Acute[b]	6	21.9 (1.4)	46.0 (5.1)	0.48	5.5 (0.5)
Chronic[c]	6	29.3 (6.1)	18.6 (2.6)	1.53	13.8 (2.2)

[a] Percentage of cells within the lymphocyte gate (SE).
[b] 6–7 days postinoculation.
[c] Maternal antibody-protected mice at 24–34 days postinoculation.

tis and die at 6–7 days p.i. When the brains of these mice were analyzed at 5 days or earlier, some inflammatory cells were apparent, but these greatly increased in number by 6 days. For this reason, we restricted our analysis to brains isolated 6 or 7 days p.i. Phenotypic analysis of CNS lymphocytes from six mice using the FACS identified 46% as CD8, 22% as CD4, and 5% as B cells (Table 1). These results are similar to previously reported results using BALB/c mice acutely infected with MHV–JHM (Williamson *et al.,* 1991). To determine the localization of these cells in the brain, sequential frozen sections were analyzed for viral RNA by *in situ* hybridization and for CD4 and CD8 cells by immunohistochemistry. By 6–7 days, MHV–JHM was detected primarily in the brainstem, although it was also present at lower levels throughout the brain (Fig. 1A). Cellular infiltrates were detected in the vicinity of MHV-infected cells around blood vessels and in the parenchyma. As shown in Figs. 1B and 1C, both CD4 and CD8 cells were detected in the parenchyma, with CD8 cells appearing more abundant, consistent with the FACS phenotyping data.

Suckling C57Bl/6 mice inoculated intranasally with MHV–JHM and nursed by dams immunized against the virus do not clear the virus from the CNS and develop a late onset, symptomatic demyelinating encephalomyelitis. Lymphocytes are also readily isolated from the brain and spinal cord of these mice and phenotypic analysis of these cells using the FACS showed that CD4 cells were more abundant than CD8 cells in these brains, in contrast to the results obtained with the acutely infected mice (Table 1). We next examined the distribution of virus and inflammatory infiltrate in mice with the chronic infection. Most of these show signs of clinical disease between 3 and 8 weeks p.i., but occasional mice become symptomatic either before 3 weeks or after 8 weeks. As shown in Fig. 2, areas of demyelination with adjacent cellular infiltrates were prominent whether mice developed disease at relatively early or later times after infection. The inflammatory cells were localized to the areas of the brain and spinal cord containing virus-infected cells, with uninfected areas lacking a significant cellular infiltrate. Both CD4 and CD8 cells were present in these infiltrates (Fig. 3), although there appeared to be relatively more CD4 cells than in the CNS of acutely infected mice, in agreement with the FACS data. In the next set of experiments, the function of the CD8 cells isolated from the CNS of infected mice was assayed.

Cytotoxic activity of lymphocytes isolated from the brain and spinal cord of MHV–JHM-infected C57Bl/6 mice

Experiments measuring anti-MHV CTL activity in C57B l/6 mice are hampered by the lack of a convenient target that is both MHC-matched and readily infected by MHV. This difficulty can be overcome by using recombinant vaccinia virus expressing the proteins of interest to infect MHC-compatible targets. The large number of nonstructural proteins believed to be encoded by the MHV genome as well as the lack of information about their structure makes this approach less practical for proteins other than the structural ones. In the next set of experiments, we used targets infected with VV expressing the S, N, or M proteins of MHV to determine the cytotoxic activity of lymphocytes isolated from the brains of infected mice. For analysis of the S protein, we used a recombinant VV which expressed a protein deleted in the transmembrane domain and therefore incapable of insertion into the plasma membrane. Cells expressing full-length S protein are lysed by MHC-incompatible effector cells and by lymphocytes isolated from uninfected MHC-compatible mice, making these cells not useful in CTL assays. This effect is most likely due to interaction of the S protein on the surface of infected cells with a component of the B cell membrane, resulting in nonspecific lysis (Welsh *et al.,* 1986). A fourth structural protein, the hemagglutinin-esterase (HE), is expressed in some strains of MHV, but cannot be detected by blot analysis when our strain of MHV–JHM was used to infect cells (E. Barnett and S. Perlman, unpublished data). Consequently it was not assayed in these experiments.

For these experiments, cells isolated from the brains of 2–4 infected mice were pooled to obtain a sufficient number of cells for the CTL assay. As shown in Fig. 4A, significant CTL activity against the S protein expressed in MC57 (H-2^b) cells could be detected when lymphocytes from the brains of acutely infected C57Bl/6 mice were analyzed. No activity was detected when VV-infected targets expressing the M or N protein were assayed. No lysis occurred when J774.1 (H-2^d) cells were used as targets (data not shown), showing that this activity was MHC-restricted. No significant CTL activity against the S, M, or N proteins was detected when spleen lymphocytes isolated from these mice were analyzed (Fig. 4B). The CTL response was CD8-specific since CTL activity was removed when effector samples

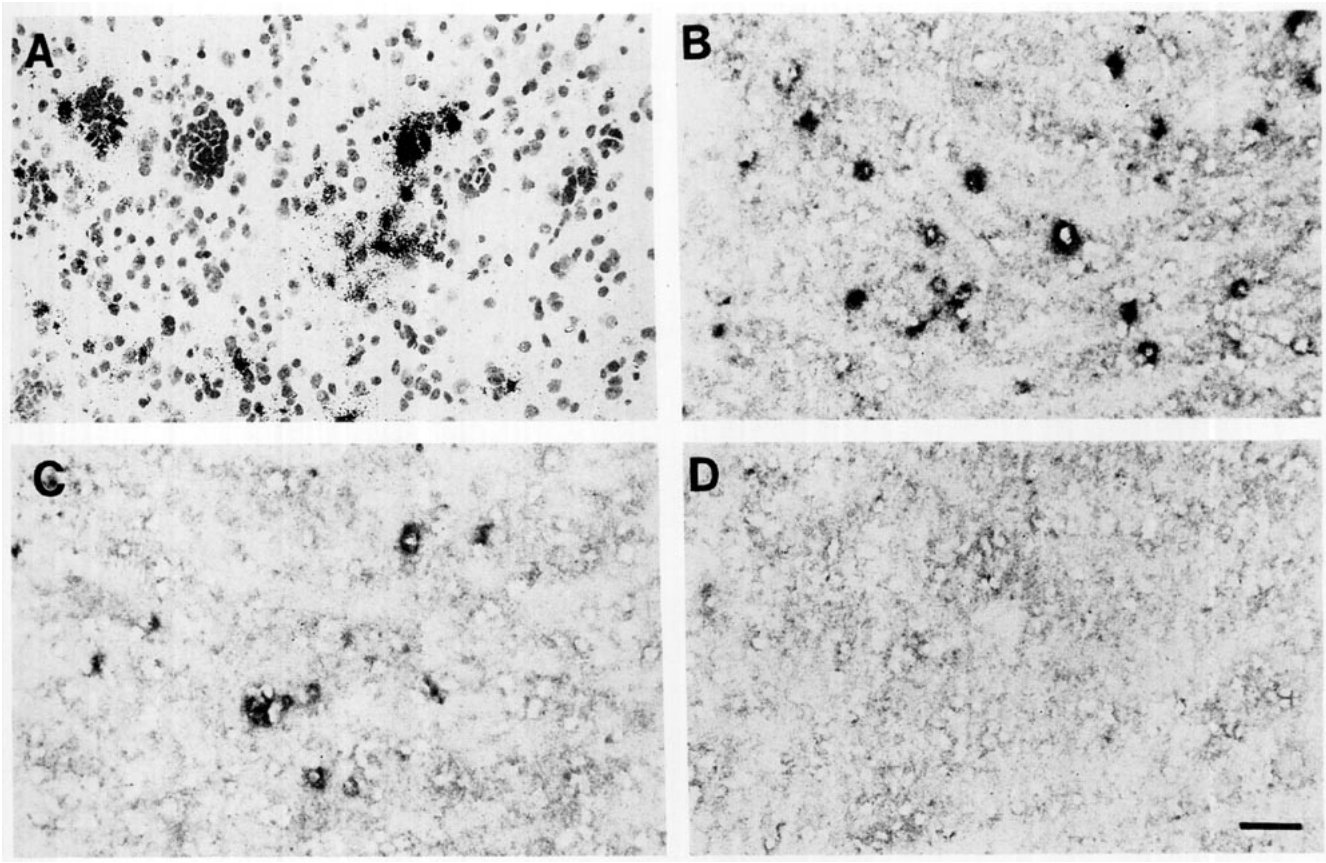

FIG. 1. Distribution of CD8 and CD4 T lymphocytes and of virus-infected cells in the brain of a mouse with acute encephalitis. A 6-week-old C57Bl/6 mouse was inoculated intranasally with MHV–JHM and the brain harvested 7 days later. Serial frozen sections were analyzed for viral RNA and CD8 and CD4 T cells as described under Materials and Methods. (A) *In situ* hybridization for MHV-specific RNA. (B) Immunohistochemical localization of CD8 cells. (C) Immunohistochemical localization of CD4 cells. (D) Staining with irrelevant antibody (negative control). Bar, 50 μm. The sections shown in B–D were fixed for only a brief period of time and were not counterstained in order to enhance detection of CD4 and CD8 staining.

were treated with anti-CD8 monoclonal antibody and complement but not with anti-CD4 antibody and complement (Fig. 5A).

A similar analysis was performed on lymphocytes isolated from the brains, spinal cords, and spleens of mice symptomatic with the late onset encephalomyelitis. The mice studied in these experiments developed hindlimb paralysis 25–35 days p.i. As in the acutely infected mice, lymphocytes isolated from the CNS exhibited cytolytic activity only against the S protein and not against the N protein (Fig. 6A). M protein was not assayed in these experiments since only a limited number of persistently infected mice were available. No CTL activity against either protein was detected in the splenic lymphocytes isolated from the chronically infected mice (Fig. 6B).

CTL activity of CNS lymphocytes isolated from mice recombinant in the H-2 locus

Suckling BALB/c mice are as susceptible to the acute encephalitis caused by MHV–JHM as C57Bl/6 mice but are protected from developing this disease if nursed by dams immunized against the virus. In marked contrast to the results obtained with the C57Bl/6 mice, these mice do not develop the late onset symptomatic encephalomyelitis (Perlman *et al.*, 1987b; Table 2). One difference between the two strains is that cytotoxic CD8 T cells active against targets expressing the nucleocapsid protein of MHV have been isolated from the brains of encephalitic BALB/c mice and from the spleens of mice inoculated intraperitoneally against MHV (Stohlman *et al.*, 1992, 1993) but not from those of similarly treated C57Bl/6 mice. In the next set of experiments, we determined, using mice congenic in the H-2 locus, whether replacing the D^b locus with the D^dL^d loci was sufficient to protect mice from the late onset symptomatic disease. Since the anti-N CTL response in BALB/c mice is known to be L^d-restricted, these congenic mice, which express L^d, should be able to mount a CTL response against the N protein. First, we assayed the brains and spleens of acutely infected BALB/c mice for anti-N cytolytic activity. As shown in Fig. 7A, CNS-infiltrating lymphocytes

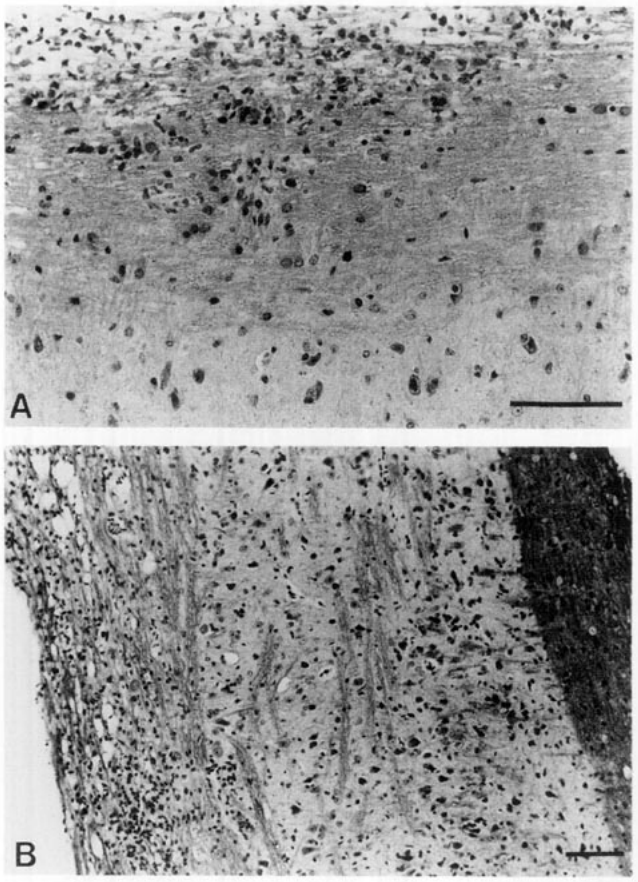

FIG. 2. Spinal cords from mice with demyelinating encephalomyelitis. Suckling C57Bl/6 mice nursed by dams immunized against MHV–JHM developed hindlimb paralysis at 17 days (A) and 46 days (B) p.i. The spinal cord and brain were fixed in formalin and embedded in paraffin. Sections (5–10 μm) were cut and stained with luxol fast blue. (A) A prominent area of demyelination with inflammatory cellular infiltrates and vacuolation is adjacent to normal-appearing white matter and gray matter. (B) Demyelination with inflammatory infiltrates is apparent on one side of the spinal cord (left) with relatively unaffected white matter present on the opposite side. Bar, 100 μm.

from BALB/c mice lyse target cells expressing the nucleocapsid protein, but not cells expressing the S or M proteins. No CTL activity was observed in spleen lymphocytes isolated from these mice against any of the proteins tested (Fig. 7B). The cytotoxic cells were phenotypically CD8 since treatment with anti-CD8 monoclonal antibody plus complement removed all the activity, whereas treatment with anti-CD4 antibody plus complement had no effect (Fig. 5B).

Next, B10.A(18R) mice, derived from C57Bl/10 mice (H-2^b) but with the d alleles of the D and L loci, were used in CTL assays. The B10.A(18R) mice are fully susceptible to the acute encephalitis caused by MHV–JHM. When lymphocytes isolated from the brains of acutely infected mice were assayed for CTL activity using J774.1 cells (H-2^d), the pattern of CTL response was identical to that observed in the BALB/c mice with only activity against the N protein detected (Fig. 7C). In addition, as in the BALB/c mice, no CTL activity against the N, S, or M proteins was observed in lymphocytes isolated from the spleens of these mice (Fig. 7D). In these experiments, we did not assay CTL activity using VV-infected MC57 cells (H-2^b), so we do not know if the recombinant mice were able to mount a K^b-restricted response against the S protein.

To prove that CD8 cells were responsible for the cytotoxic activity which we detected in the B10.A(18R) mice, CD4 and CD8 cells were selectively eliminated using antibody and complement. As shown in Fig. 5C, lysis of CD4 cells did not decrease the cytotoxic activity of the brain lymphocytes. Similar treatment with antibody directed against CD8 cells virtually eliminated all of this activity, showing that the cytotoxic cells were contained within this fraction.

Late onset demyelinating encephalomyelitis in B10.A(18R) mice

To determine whether the B10.A(18R) mice develop the late onset encephalomyelitis, three females were immunized against MHV–JHM and their offspring inoculated intranasally with MHV–JHM. As a control for these experiments, we showed that C57Bl/10 mice developed the late onset disease at a rate similar to that observed in C57Bl/6 mice (Table 2). Nursing by immunized dams prevented the acute encephalitis in the recombinant mice, but 16% of the mice developed the late onset disease (Table 2). This rate is lower than in the C57Bl/6 and C57Bl/10 strains (Table 2) but, unlike the BALB/c mice, some of the congenic mice do develop hindlimb paralysis. The distribution of viral RNA and CD4 and CD8 cells in the brains and spinal cords of these mice is indistinguishable from that observed in the C57Bl/6 mice (data not shown), suggesting that the pathogenesis of the disease is the same in both types of mice.

DISCUSSION

The pathogenesis of the demyelination induced by MHV–JHM is a complex process, most likely involving both host and virus components. Several studies show that different strains or mutants of MHV–JHM can cause either acute encephalitis or demyelination to variable degrees and some of these mutations have been mapped to the S protein (Parker *et al.*, 1989; Wang *et al.*, 1992). The role of the immune system in the disease is more controversial, since some studies suggest that the demyelinating process has no immune component (Lampert *et al.*, 1973; Weiner, 1973; Knobler *et al.*, 1982; Love *et al.*, 1987), whereas others show that the demyelination is largely immune-mediated (Wang *et al.*, 1990). These apparently contradictory results are in part explained by the different strains

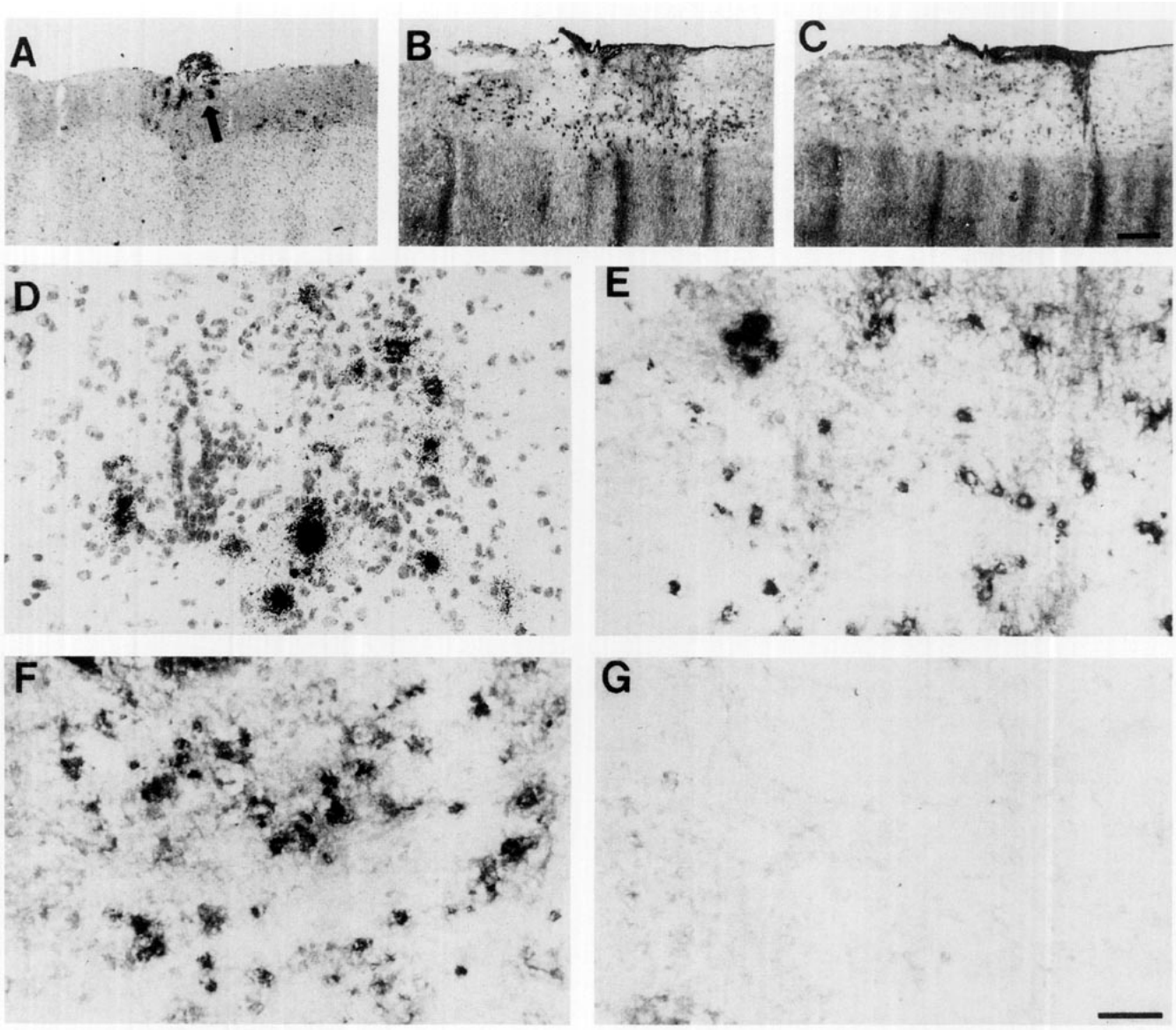

FIG. 3. Distribution of CD8 and CD4 T cells in the brain of a chronically infected mouse. A C57Bl/6 mouse nursed by a dam immunized against MHV–JHM developed hindlimb paralysis at 45 days p.i. The brain and spinal cord were harvested and serial frozen sections assayed as described in Fig. 1. (A and D) *In situ* hybridization for MHV RNA. (B and E) CD8 cells. (C and F) CD4 cells. (G) Staining with irrelevant antibody (negative control). The sections shown in B and C and E–G were fixed for only a brief period of time and were not counterstained in order to enhance detection of CD4 and CD8 staining. Bar: A–C, 250 μm; D–G, 50 μm.

of mice and virus and different protocols used in these reports.

In the maternal antibody-protected model, neurons are not infected to a significant extent a few days p.i. and thus the mice do not develop encephalitis, although virus moves transneuronally to reach the spinal cord (Perlman *et al.*, 1990b). Over the next several weeks, a variable percentage then develop a demyelinating encephalomyelitis, characterized by extensive inflammatory infiltrates in the white and to a lesser extent the gray matter (Fig. 2). Our results show that this process is at least partially host-dependent since both C57Bl/6 and BALB/c mice uniformly develop acute encephalitis, although only C57Bl/6 mice develop a late onset symptomatic demyelinating disease (Table 2). In this model, active virus replication appears to be important, since virus can be detected only in symptomatic mice, although viral antigen can be detected in all C57Bl/6 mice whether symptomatic or not (Perlman *et al.*, 1987a). Our results also show that the brains of symptomatic, persistently infected C57Bl/6 mice, like those of acutely infected mice, contain CD8 lymphocytes which are cytotoxic for cells expressing the S protein. Splenic CD8 cells with activity against the S protein have also been detected when C57Bl/6 mice inoculated intraperitoneally with MHV–JHM were analyzed in secondary CTL assays (S. Stohlman, personal communication). The inability of these cells to effect

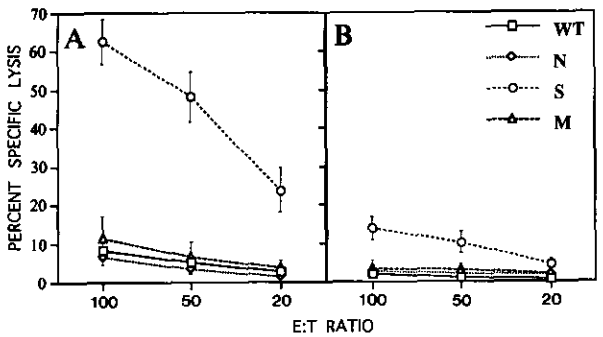

FIG. 4. CTL activity in C57Bl/6 mice with acute encephalitis (6–7 days p.i.). MC57 cells were infected with recombinant VV expressing M, N, or S and labeled with ^{51}Cr at 6 hr p.i. The percentage specific ^{51}Cr release was determined after incubation with lymphocytes derived from the brains (A) and spleens (B) of infected mice. Cells isolated from 2–4 mice were used in each experiment. Each point represents the mean of percent specific lysis for all the experiments. Bars show the standard error for each point.

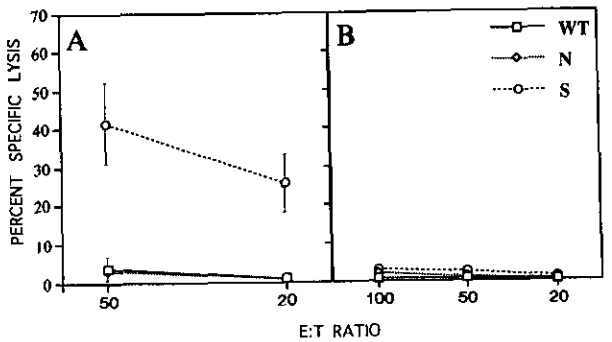

FIG. 6. CTL activity in persistently infected C57Bl/6 mice. Suckling mice were nursed by immunized dams and inoculated with virus at 10 days. Mice developed hindlimb paralysis at 25–35 days p.i. In each experiment, lymphocytes from the brain and spinal cord (A) and spleen (B) of 2–4 mice were analyzed for anti-MHV CTL activity using MC57 cells infected with recombinant VV expressing the N or S proteins. Each point represents the mean of percent specific lysis for all experiments. Bars show the standard error for each point.

virus clearance may reflect a quantitative deficiency in the number of anti-S CTLs present. Alternatively, it is also possible that these cells contribute to the demyelinating process, although this has not been proven. Of note, class I MHC antigens can be detected on oligodendrocytes isolated from MHV-infected mice (Suzumura *et al.*, 1986). Such cells, if also infected with MHV, would presumably be a target for anti-MHV CTL activity.

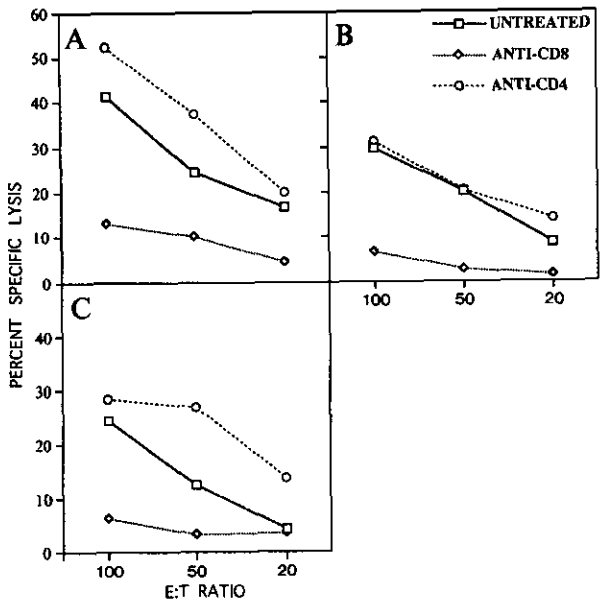

FIG. 5. Complement depletion of CD4 and CD8 brain lymphocytes. Cells were isolated from the brains of mice with acute encephalitis. Prior to incubation with targets, the effectors were treated with anti-CD8 or anti-CD4 plus complement as described under Materials and Methods. MC57 cells expressing the S protein were used in A, whereas J774.1 cells expressing the N protein were used in B and C. (A) Brain lymphocytes from C57Bl/6 mice. (B) Brain lymphocytes from BALB/c mice. (C) Brain lymphocytes from B10.A(18R) mice.

Several explanations exist for the observed host differences. Even though one report suggested that MHV-infected BALB/c mice develop more splenic dysfunction as measured by proliferative response to concanavalin A than do the C57Bl/6 mice (Smith *et al.*, 1991), virus may be cleared more completely in maternal antibody-protected BALB/c mice during the primary infection. As a consequence, little or no replication-competent virus would remain in these animals and the late onset increase in virus replication could not occur as it does in C57Bl/6 mice. Consistent with this interpretation, less viral RNA is detected in asymptomatic, maternal antibody-protected BALB/c mice at early times p.i. than in C57Bl/6 mice when assayed by *in situ* hybridization (E. Barnett and S. Perlman, unpublished observations). Alternatively, the two strains of mice may clear the virus equally well during the initial stages of the infection, but the C57Bl/6 mice may be unable to suppress the amplification of the virus that occurs at the time when the mice develop hindlimb paralysis.

Previously published data are also consistent with the notion that MHV–JHM more commonly causes a

TABLE 2

NUMBER OF MICE WITH HINDLIMB PARALYSIS AND MATERNAL ANTIBODY TITERS

Strain	Number with hindlimb paralysis/Total (%)	Maternal antibody titer (No.)[b]	Range of titers
C57Bl/6	162/249 (65)	1:1655 (19)	1:380–1:7200
C57Bl/10	17/38 (45)	1:1753 (4)	1:100–1:2080
BALB/c[a]	0/43 (0)	1:3269 (7)	1:1174–1:5754
B10.A(18R)	7/43 (16)	1:1821 (3)	1:863–1:2500

[a] Includes mice described previously (Perlman *et al.*, 1987b).
[b] Number of mice averaged.

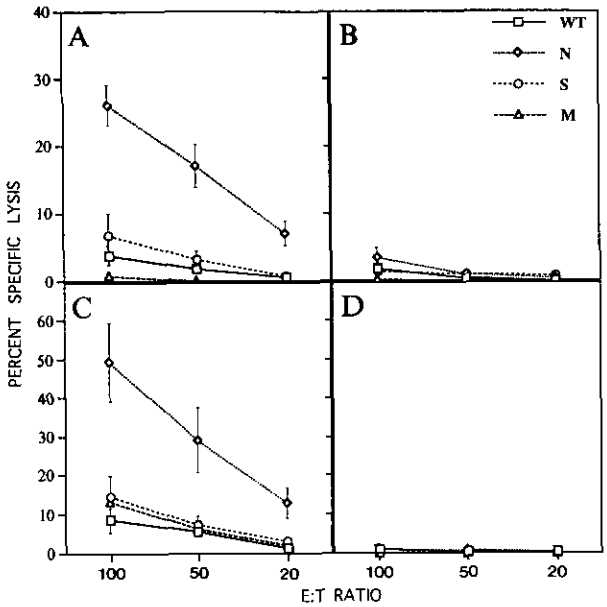

FIG. 7. CTL activity in BALB/c and B10.A(18R) mice with acute encephalitis. J774.1 cells were infected with recombinant VV expressing M, N, and S proteins for 6 hr prior to labeling with ^{51}Cr. The percentage specific ^{51}Cr release was determined after incubation with lymphocytes isolated from the brains (A and C) and spleens (B and D) of BALB/c (A and B) or B10.A(18R) (C and D) mice. Each point represents the mean of percent specific lysis for all experiments. Bars show the standard error for each point.

demyelinating disease in C57Bl/6 than in BALB/c mice. Stohlman and Weiner reported that on histological examination of infected brains, 46% of C57Bl/6 mice but only 15% of the BALB/c mice contained evidence of demyelination (Stohlman and Weiner, 1981). In addition, we have observed that intranasal inoculation with ts8, a mutant of MHV–JHM which causes demyelination without a significant amount of encephalitis (Haspel *et al.*, 1978), resulted in more extensive disease in C57Bl/6 than in BALB/c mice. Thus, histological examination of the brains of BALB/c mice at 18–27 days p.i. revealed a few areas of spongiform degeneration without a significant inflammatory response, whereas the brains of C57Bl/6 mice similarly infected and analyzed revealed larger areas of demyelination with a substantial inflammatory component (R.C., unpublished observations).

We could not detect a significant response to the S protein in the spleens of acutely or persistently infected C57Bl/6 mice even though such a response was present in the brains of both types of mice (Figs. 4 and 6). Similarly, N-specific CTL activity was present in the brain, but not the spleen ((Stohlman *et al.*, 1993), Figure 7) or the cervical lymph node (Stohlman *et al.*, 1993) of BALB/c mice with acute encephalitis. Previously we have reported that CD4 cells able to proliferate in response to the S protein were present in the spleens both of acutely and persistently infected C57Bl/6 mice and of mice immunized intraperitoneally against MHV–JHM, but an M-specific response was present only in the immunized mice. The explanation for these apparent differences in lymphocyte response or trafficking remains to be determined.

Our results indicate that the lack of a CTL response to the nucleocapsid protein is not the sole explanation for the propensity for C57Bl/6 mice to develop the late onset demyelinating disease. A lower percentage of mice developed hindlimb paralysis in the mice recombinant at the H-2 locus, suggesting that the CTL response to the N protein or to another MHV protein presented by the D^d or L^d protein decreases the likelihood of developing the late onset disease. Our experiments also do not eliminate the possibility that susceptibility to the late onset disease maps entirely to the class I locus, since the B10.A(18R) mice do not contain the K^d allele.

Previous studies have revealed that CD4 cells are also necessary for MHV–JHM clearance from the brains of infected mice and rats (Williamson and Stohlman, 1990; Körner *et al.*, 1991). In at least one instance, MHV-specific CD4 cells can effect virus clearance in the apparent absence of CD8 cells (Körner *et al.*, 1991). In another model of demyelination, that induced by Theiler's virus, CD4 cells have a role in the demyelinating process and anti-CD4 therapy reduces the severity of the disease (Rodriguez *et al.*, 1986; Friedmann *et al.*, 1987; Welsh *et al.*, 1987). C57Bl/6 mice, and presumably B10.A(18R) mice, mount a CD4 response to the M and S proteins (Mobley *et al.*, 1992). Whether the CD4 response in the C57Bl/6 mice contributes to the pathogenesis of the demyelinating process is at present under investigation.

Genetic analysis of mice infected with Theiler's virus shows that susceptibility is multifactorial and includes a gene in the H-2D region as well as loci on other chromosomes (Bureau *et al.*, 1993). Previous studies of the genetic basis of susceptibility to MHV–JHM infections have shown that resistance to the acute encephalitis is controlled by one or two genes, one of which maps to chromosome 7 (Stohlman and Frelinger, 1978; Knobler *et al.*, 1984; Smith *et al.*, 1984). Little information is available about the genetic basis of susceptibility to the late onset demyelinating disease. In one recent study, Kyuwa *et al.* examined the genetic basis of the late onset vacuolar degeneration which develops in STS/A mice infected with MHV–JHM. No single genetic locus could be identified, suggesting that susceptibility to this late onset disease is multifactorial (Kyuwa *et al.*, 1992). These results may be relevant for MHV–JHM-induced demyelination, although the vacuolar degeneration observed in the STS/A mice is different from the demyelinating process observed in our model. It should be possible to determine, using recombinant strains of mice, whether the genetic basis of suscepti-

bility to MHV–JHM-induced demyelination is also multifactorial.

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