Macrophage Infiltration, but Not Apoptosis, Is Correlated with Immune-Mediated Demyelination following Murine Infection with a Neurotropic Coronavirus

GREGORY F. WU¹ AND STANLEY PERLMAN^{1,2,3*}

Program in Neuroscience¹ and Departments of Pediatrics² and Microbiology,³ University of Iowa, Iowa City, Iowa 52242

Received 19 April 1999/Accepted 30 June 1999

Mice infected with mouse hepatitis virus strain JHM (MHV-JHM) develop a chronic demyelinating encephalomyelitis that is in large part immune mediated. Potential mechanisms of immune activity were assessed using an adoptive transfer system. Mice deficient in recombinase-activating gene function (RAG1^{-/-}), defective in B- and T-cell maturation, become persistently infected with MHV but do not develop demyelination. Adoptive transfer of splenocytes from mice immunized to MHV into RAG1^{-/-} mice infected with an attenuated strain of the virus results in the rapid and progressive development of demyelination. Most striking, adoptive transfer resulted, within 5 to 6 days, in extensive recruitment of activated macrophages/microglia to sites of demyelination within the spinal cord. Clearance of virus antigen occurred preferentially from the gray matter of the spinal cord. Apoptotic cells were identified in both the gray and white matter of the central nervous system (CNS) from RAG1^{-/-} mice before and after adoptive transfer, with a moderate increase in number, but not distribution, of apoptotic cells following the development of demyelination. These results suggest that apoptosis following MHV-JHM infection of the murine CNS is not sufficient to cause demyelination. These results, showing that macrophage recruitment and myelin destruction occur rapidly after immune reconstitution of RAG^{-/-} mice, suggest that this will be a useful system for investigating MHV-induced demyelination.

Mouse hepatitis virus (MHV) strain JHM (MHV-JHM) is a neurotropic coronavirus which causes both acute and chronic infections of the central nervous system (CNS) in susceptible rodents (15, 19, 22). Intranasal inoculation of C57BL/6 (B6) mice results in a fatal acute encephalitis around 5 to 7 days postinoculation (p.i.). Several experimental strategies have been developed to protect mice from acute disease (15). In one model, suckling mice are protected by nursing dams immunized to MHV. After intranasal inoculation with virus, they do not develop acute disease. However, a variable percentage (40 to 90%) develop a chronic persistent infection of the CNS which results in demyelination and hindlimb paralysis at 3 to 8 weeks p.i. (38). In another model, direct intracranial inoculation with an attenuated variant of MHV-JHM, J2.2-v1, results in mild acute disease which resolves, giving rise to a chronic state of CNS demyelination evidenced clinically by hindlimb weakness at around 10 to 12 days p.i. (9, 48). The pathological similarities that MHV-JHM-induced demyelination shares with multiple sclerosis (MS) make it a useful experimental model for this human demyelinating disease.

The pathogenesis of MHV-JHM-induced CNS disease is a result of a balance between viral infection and host immune response (15). Although the issue remains controversial to a degree, demyelination following infection with MHV-JHM appears to be in large part immune mediated. Experiments involving J2.2-v1 infection of mice with severe combined immunodeficiency (SCID) showed that in the absence of T lymphocytes, viral infection of the CNS did not result in demyelination (49). However, demyelination developed only if

Thy1.1⁺ lymphocytes were adoptively transferred to these MHV-infected SCID mice (10).

Although T lymphocytes have been implicated in the induction of demyelination following infection with MHV, specific downstream mechanisms of immune-mediated pathogenesis have not been clearly defined. Neither perforin-mediated cytotoxicity nor gamma interferon (IFN- γ) is required for the development of MHV-JHM-induced demyelination (25, 37). Therefore, other cellular immune responses, e.g., Fas-mediated apoptosis or other proinflammatory cytokine-mediated damage, are potential mechanisms of immune-mediated demyelination following MHV-JHM infection of the murine CNS.

The induction of macrophage infiltration and activation in relation to demyelination in MS and experimental animals suggests a direct role for these cells in the effector phase of demyelination. Demyelinating lesions in the CNS of MS patients contain large quantities of macrophages, particularly surrounding plaque borders (4, 5). A large quantity of activated macrophages has been observed in MHV-JHM-induced lesions (20, 44). Furthermore, depletion of blood-borne macrophages prevents experimental allergic encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus (TMEV)-induced demyelination but not MHV-induced demyelination (16, 39, 46, 50). Macrophages are therefore a common element in the pathology of CNS demyelination. The proposed mechanisms by which macrophages may be directly involved in destruction of myelin include not only mechanical removal of myelin by phagocytosis but also the secretion of cytokines and toxic molecules which have been shown to damage oligodendrocytes (42).

One potential result of immune activation following MHV infection of the CNS is the targeted induction of a cascade of events known as apoptosis. The contribution of apoptosis in animals with experimentally induced demyelination is contro-

* Corresponding author. Mailing address: Department of Pediatrics, University of Iowa, 2042 Medical Labs, Iowa City, IA 52242. Phone: (319) 335-8549. Fax: (319) 335-8991. E-mail: Stanley-Perlman@uiowa.edu.

versal. In many reports, a majority of apoptotic cells have been identified as T lymphocytes, with the hypothesis that apoptosis serves to clear specific and nonspecific lymphocytes following infiltration into the CNS (2, 11). However, some recent data suggest that apoptosis contributes to clinical disease and demyelination in mice with EAE, since disease is much milder in *lpr* and *gld* mice lacking the Fas and Fas ligand molecules, respectively (41, 47). The degree of apoptosis in these animals is disproportionately diminished relative to the degree of inflammation, indicating that Fas-mediated apoptosis may play a direct role in the destruction of resident CNS cells.

The role of apoptosis in the pathogenesis of MS has been actively investigated and remains questionable. Although apoptosis is observed associated with demyelinating lesions in the CNS of patients with MS, in one report, apoptosis was not detected in oligodendrocytes. Rather, a majority of apoptosis occurred in lymphocytes, even though oligodendrocyte expression of Fas was elevated (3). Although oligodendrocyte damage was seen following Fas cross-linking in vitro, apoptosis of oligodendrocytes was not detected, suggesting that oligodendrocyte apoptosis may not be the mechanism of immune-mediated damage to myelin in MS (7). On the other hand, in a study by Dowling et al., up to 40% of apoptotic cells in post-mortem chronic MS lesions labeled by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) were identified as oligodendrocytes, while minimal T-cell apoptosis was detected (6).

T-cell and oligodendrocyte apoptosis have also been observed in MHV-JHM-infected Lewis rats with subacute demyelinating encephalomyelitis (1). In these animals, the quantity of T-cell apoptosis was always greater than the quantity of oligodendrocyte apoptosis; furthermore, oligodendrocyte necrosis was also seen. In perforin-deficient mice irradiated prior to infection with J2.2-v1, apoptosis was virtually eliminated, suggesting that the apoptosis seen following infection was lymphocyte mediated (25).

To investigate potential mechanisms involved in MHV-JHM-induced demyelination, an adoptive transfer system using MHV-infected mice deficient in recombinase-activating gene function (*RAG1*^{-/-}) was analyzed for both the level of macrophage/microglial involvement and the pattern of apoptosis. While apoptosis does not appear to play a key role in MHV-JHM-induced demyelination, our data strongly support a role for macrophages in this process. Our results show that increased numbers of macrophages are detected in the infected CNS shortly after the adoptive transfer of splenocytes and coincident with the development of demyelination.

MATERIALS AND METHODS

Viruses. MHV-JHM was grown and titered on BALB/c 17c1-1 cells as previously described (38). A neuroattenuated variant of MHV-JHM, J2.2-v1 (9), was generously provided by J. Fleming (University of Wisconsin, Madison).

Animals. Pathogen-free B6 mice were obtained from the National Cancer Institute (Bethesda, Md.). *RAG1*^{-/-} mice on a B6 background were obtained from The Jackson Laboratory (Bar Harbor, Maine) and bred at the University of Iowa. No mature T or B cells are produced in *RAG1*^{-/-} mice, but NK and macrophage cell quantity and function are normal.

Experimental paradigms. Several experimental models of MHV infection of the CNS were used in this study. (i) To obtain acutely infected mice, 6-week-old B6 mice were inoculated with 4×10^4 to 6×10^4 PFU of MHV-JHM intranasally. (ii) B6 mice persistently infected with MHV-JHM were generated by inoculating 10-day-old B6 mice with 4×10^4 to 6×10^4 PFU of MHV-JHM intranasally and nursing with immunized dams, as described previously (38). (iii) In most experiments, *RAG1*^{-/-} mice (n = total of 29) were infected with J2.2-v1 by intracranial injection of 10^3 PFU diluted in 30 μ l of Dulbecco modified Eagle medium (DMEM) with 15 mM HEPES (pH 7.0) (49). In some cases (n = 15 mice), these mice were the recipients of adoptively transferred splenocytes from immunized animals as described below. To generate *RAG1*^{-/-} mice persistently infected with the wild-type MHV-JHM, 6-week-old *RAG1*^{-/-} mice were given a

50 μ l-50 μ l mixture of two anti-S neutralizing antibodies, 5A13.5 (neutralizing titer, 1:45,000) and 5B19.2 (neutralizing titer, 1:2,700) (both kindly provided by M. Buchmeier, The Scripps Research Institute) by intraperitoneal inoculation immediately prior to infection with virus. This treatment prevents acute encephalitis, and *RAG1*^{-/-} mice infected in this way become symptomatic at approximately 30 to 40 days p.i.

Preparation of splenocytes for adoptive transfer. Donor spleen cells were harvested from B6 mice 6 days following intraperitoneal immunization with live virus (3×10^5 PFU of MHV-JHM in 500 μ l of phosphate-buffered saline). Spleens were mechanically disrupted in 5 ml of DMEM, triturated, and then filtered through nylon mesh. After lysis of erythrocytes (18) and washing in DMEM supplemented with 5% fetal calf serum, 5×10^6 to 1×10^7 cells were delivered in 500 μ l of DMEM via injection into the retro-orbital sinus. To determine if any infectious virus was transferred coincidentally with the splenocytes, 5×10^6 spleen cells from three individual mice were assayed by plaque assay on 17c1-1 cells. No infectious virus was detected.

Histology. Mice were killed by sodium pentobarbital overdose and transcardially perfused with phosphate-buffered saline. Brains and spinal cords were removed and placed in 10% normal buffered formalin for 2 days at room temperature (RT) and then embedded in paraffin. All sections were cut at 8 μ m. For examination of myelin and cell morphology, sections were cut, processed, stained with luxol fast blue (LFB), and counterstained with hematoxylin and eosin.

TUNEL. Detection of in situ DNA fragmentation was done with a fluorescein in situ death detection kit (Boehringer Mannheim, Indianapolis, Ind.) as specified by the manufacturer.

Double labeling. Double labeling for TUNEL with virus antigen or macrophages was accomplished as follows. After completion of the TUNEL reaction, sections were permeabilized with 0.1% Triton X-100 and then blocked with 10% normal goat serum for 2 h at RT. After rinsing, a 1:2,000 dilution of monoclonal antibody 5B188.2, recognizing the N protein of MHV-JHM (provided by M. Buchmeier), or a 1:50 dilution of rat anti-mouse F4/80 (CI:A3-1; Serotec, Oxford, England) was added, and sections were incubated overnight at 4°C. F4/80 recognizes a macrophage-specific protein with homology to a family of hormone receptors (28). After washing, sections stained for virus antigen were incubated with Texas red-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove, Pa.) for 1 h. Sections stained for macrophages were incubated with biotinylated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories) for 1 h at RT followed by treatment with avidin-conjugated horseradish peroxidase (Jackson Immunoresearch Laboratories) and then 3,3'-diaminobenzidine (Sigma, St. Louis, Mo.) as the final substrate during development.

A slightly different protocol was used to detect apoptotic astrocytes. Because of difficulty in maintaining antigenicity of the astrocyte-specific protein, glial fibrillary acidic protein (GFAP), immunohistochemical labeling of GFAP was performed followed by the TUNEL reaction. Sections were hydrated and permeabilized and then treated with CAS block (Zymed, San Francisco, Calif.) for 10 min at RT. Monoclonal anti-GFAP (Sigma) was added, and sections were stained as described above for viral antigen. After washing of secondary antibody, the TUNEL protocol was followed as instructed by the manufacturer. All fluorescently labeled sections were coverslipped with Vectashield (Vector, Burlingame, Calif.). No TUNEL-positive nuclei were seen in sections from uninfected animals or if terminal deoxynucleotidyltransferase enzyme was omitted. No immunolabeling was seen with the omission of primary antibody.

Laddering assay. Gel electrophoretic detection of DNA fragmentation in the CNS of MHV-JHM-infected mice was done as previously described (35). Briefly, either one hemisphere of the cerebrum or one olfactory bulb was homogenized in 10 volumes of Tris-EDTA buffer. EDTA and Triton X-100 were brought to concentrations of 10 mM and 0.5%, respectively. The sample was vortexed and then centrifuged at $13,000 \times g$ for 10 min. The supernatant was incubated overnight at 37°C with 0.5% sodium dodecyl sulfate and 0.1 μ g of proteinase K per ml and then extracted with phenol and chloroform. Nucleic acid material (15 μ g) was treated with RNase prior to analysis on a 1% agarose gel.

Imaging. Samples assayed for apoptosis by TUNEL staining alone or double labeled for TUNEL and viral antigen or for TUNEL and GFAP were imaged in a Bio-Rad MRC-1024 krypton-argon scanning laser confocal microscope. Slides stained for macrophage/TUNEL double labeling, or with LFB and/or hematoxylin and eosin, were imaged in a Leitz diaphan fluorescent/light microscope equipped with an Optiphot charge-coupled device camera for digitalization. Matrox Inspector software (Matrox Imaging, Montreal, Quebec, Canada) was used to capture the images. Quantification of TUNEL labeling was done by evaluating the number of TUNEL-positive nuclei per section. Section area was then measured by tracing of digital images with VTrace software (Image Analysis Facility, University of Iowa). Quantitation of TUNEL labeling is expressed as the number of TUNEL-positive nuclei per area of section, and numbers are an average of at least three independent sections of tissue. Quantification of macrophage density was done in a similar manner, with the number of F4/80-positive cells in a tissue section divided by the area of that section as measured by VTrace. All acquisition of images was done at the University of Iowa Central Microscopy Research Facility. Manipulation of images and quantification of demyelination and TUNEL-positive nuclei were performed at the University of Iowa Image Analysis Facility.

RESULTS

MHV-JHM-induced demyelination correlates with gray matter clearance and occurs only in the presence of lymphocytes. Demyelination cannot be detected in MHV-infected SCID mice unless they receive splenocytes from naive or immunized mice (14, 49). To determine the relationship of macrophage infiltration and apoptosis to demyelination, immunodeficient mice were infected with wild-type MHV-JHM or its attenuated variant, J2.2-v1. $RAG1^{-/-}$ mice were used in lieu of SCID mice because the block to lymphocyte ontogeny is not leaky, as occasionally occurs in SCID mice (34). The absence of $CD4^{+}$ and $CD8^{+}$ T lymphocytes was confirmed by cell sorter analysis of $RAG1^{-/-}$ splenocytes for CD4 and CD8 (29) (data not shown).

Initial experiments involved the intranasal inoculation of $RAG1^{-/-}$ mice with wild-type MHV-JHM since this virus causes robust demyelination in immunocompetent mice. Mice were protected from acute encephalitis by intraperitoneal injection of a mixture of two anti-S neutralizing antibodies as described in Materials and Methods. These mice remained asymptomatic for at least 23 days, developing acute symptoms without evidence of demyelination anywhere from 23 to 47 days p.i. Intravenous adoptive transfer of splenocytes from naive or immunized syngeneic B6 mice occasionally resulted in hindlimb paralysis and demyelination of the spinal cord. However, in a majority of these animals, virus was cleared and mice remained asymptomatic, or clearance was incomplete and mice died of delayed neuronal disease. To develop a more reproducible model of demyelination, an experimental design similar to that of Wang et al. (49) was established by using $RAG1^{-/-}$ mice inoculated with J2.2-v1 as recipients and spleen cells from syngeneic B6 mice as donors. $RAG1^{-/-}$ mice inoculated with J2.2-v1 did not begin exhibiting clinical symptoms of neurological disease until 12 days p.i.; however, a majority of animals developed hunching, ruffling of fur, and rotational motor behavior around 15 days p.i. No evidence of demyelination was seen in spinal cord sections stained with LFB (Fig. 1A), even though viral infection was extensive. Viral titers were in the range of 10^5 PFU/g by 15 days p.i. (Table 1), and staining of virus antigen by immunohistochemistry revealed extensive viral infection of the spinal cords of symptomatic mice (Fig. 1B). Virus antigen was clearly distributed throughout both the gray and white matter of spinal cords from these mice, revealing significant neuronal infection (Fig. 1b, arrowhead) as well as extensive white matter involvement.

The adoptive transfer protocol was varied to determine the optimal donor cell quantity and incubation period for the consistent reproduction of significant demyelination and clinical disease. At day 3 or 6 p.i., 10^6 , 5×10^6 , 10^7 , and 5×10^7 cells from immunized or naive mice were administered to $RAG1^{-/-}$ mice. Transfer of splenocytes from unimmunized mice resulted in demyelination inconsistently. In contrast, demyelination, along with the rapid and obvious appearance of clinical disease, occurred following adoptive transfer of between 5×10^6 to 5×10^7 cells from immunized donors on day 3 p.i.

Therefore, in subsequent experiments, $RAG1^{-/-}$ mice infected with J2.2-v1 were given 5×10^6 to 1×10^7 immunized donor cells intravenously on day 3 p.i. Beginning at day 5 posttransfer (p.t.), mice exhibited the same clinical symptoms as occur in immunocompetent B6 mice infected with J2.2-v1, including wobbly gait and mild hindlimb paralysis (9). By day 8 p.t., mice displayed more severe clinical deficits, with virtually all adoptive transfer recipients demonstrating hindlimb paresis or paralysis. Histological examination of the spinal cord (Fig. 1E) revealed demyelination resembling that induced in the

spinal cords of wild-type B6 mice by MHV-JHM (Fig. 1C) and J2.2-v1 (data not shown). Immune infiltrates were localized to areas of demyelination, as has previously been described (38, 48). The burden of virus in these animals was distinctly less than that of J2.2-v1-infected $RAG1^{-/-}$ mice, as shown by immunohistochemical detection of viral antigen (Fig. 1D and F) and the presence of lower levels of infectious virus (Table 1). Furthermore, viral antigen was almost entirely eliminated from the gray matter following adoptive transfer. These results, in agreement with the results of Houtman and Fleming (14), clearly demonstrate that demyelination following MHV-JHM infection is mediated by immune cells and does not correlate with the quantity of virus in the CNS. However, clearance of MHV from the CNS is preferentially achieved for the gray matter after the addition of cells. The combination of infiltrating splenocytes and MHV-JHM within the CNS results in demyelination, albeit by an unidentified mechanism.

An increase in macrophages correlates with demyelination. Previous results suggest that the recruitment and activation of macrophages/microglia into sites of viral infection are major components of the host response to MHV (20, 44). To assess the role of macrophages/microglia in demyelination, we examined macrophage/microglial distribution and quantity in J2.2-v1-infected $RAG1^{-/-}$ mice before and after adoptive transfer. A moderate number of macrophages was seen throughout the gray and white matter in $RAG1^{-/-}$ mice infected with J2.2-v1, and this number remained relatively unchanged over time (see Fig. 3). In comparison, spinal cord sections from splenocyte recipients revealed many more F4/80-positive macrophages throughout the gray and white matter (Fig. 2B). The abundant increase in macrophage/microglial labeling localized directly to areas of demyelination, as revealed by serial sections stained with F4/80 immunohistochemistry and LFB (Fig. 2A, arrowheads). Furthermore, there was a distinct change in morphology of cells labeled by F4/80 primary antibody from cells with spiny ramifications (Fig. 2C), suggestive of microglia, to rounded cells consistent with an activated state (Fig. 2D).

To more precisely correlate the quantity of macrophages with the development of demyelination, macrophage density over time following adoptive transfer was measured. Quantification of macrophages at days 2, 4, and 6 p.t. revealed a sevenfold increase in macrophages (Fig. 3) within a period of 4 days. In summary, the tremendous increase in number of macrophages/microglia in the spinal cords of mice with demyelination compared to $RAG1^{-/-}$ mice without demyelination suggests that the infiltration and activation of macrophages correlate with the development of demyelination.

MHV-JHM infection of the CNS results in apoptosis. One possible mechanism of MHV-JHM-induced CNS disease is the loss of cells by apoptosis. The question of whether apoptosis is related to virus or disease or both was addressed initially by TUNEL staining of CNS tissue from B6 mice acutely or chronically infected with virulent MHV-JHM. Examination of brains from mice with acute encephalitis stained with TUNEL demonstrated the presence of apoptotic nuclei in anatomically distinct regions in the vicinity of virus-infected cells. At day 7 p.i., TUNEL-positive nuclei were seen in multiple areas of the brain, including the hippocampus, brainstem, and cerebral cortex (data not shown). TUNEL staining in combination with immunohistochemistry for viral antigen revealed numerous apoptotic nuclei in close proximity to cells infected with MHV-JHM, with virtually no overlap (Fig. 4A). A few apoptotic cells were virally infected, but this was an infrequent occurrence (Fig. 4B).

To verify that the TUNEL-positive cells were apoptotic, cells in regions of TUNEL labeling were examined for morpholog-

FIG. 1. MHV-JHM-induced demyelination occurs only in the presence of lymphocytes. $RAG1^{-/-}$ mice infected with J2.2-v1 showed no evidence of demyelination in the spinal cord (A) yet harbored large quantities of virus, as shown by immunohistochemical staining for the JHM N protein (B). Virus antigen was localized to both the white and gray matter of the spinal cord, with prominent neuronal infection (arrowhead). Normal B6 mice chronically infected with wild-type MHV-JHM exhibited areas of demyelination with inflammatory infiltrate (C, arrowheads) and persistence of virus antigen almost exclusively in the white matter (D). $RAG1^{-/-}$ mice infected with J2.2-v1 and receiving 5×10^6 immunized syngeneic spleen cells on day 3 p.i. developed demyelination in the spinal cord around day 6 p.i. (E, arrowheads). Virus was preferentially cleared in these animals from the gray matter, but antigen persisted in the white matter (F). (a, c, and e) LFB; (b, d, and f) immunohistochemical labeling for MHV nucleocapsid protein. Sections are magnified 12.5 $\times$.

ical characteristics of apoptosis, including cell shrinkage, chromatin condensation, and membrane budding (17). High-magnification images of sections stained with hematoxylin and eosin showed cells with abnormal nuclear morphology, including nuclear condensation and distortion (Fig. 4B, inset). Gel electrophoretic analysis of DNA from the CNS of MHV-JHM-infected animals was done to further confirm the presence of apoptosis in the CNS. DNA was extracted from the olfactory bulbs of infected and uninfected B6 mice and electrophoresed. A characteristic oligosomal laddering pattern was seen in DNA isolated from the MHV-JHM-infected animal. DNA from the CNS of uninfected animals was not fragmented in this manner (Fig. 4D). These results show that apoptosis occurs to a significant extent in mice with MHV-JHM-induced CNS disease

in the general locale of infected sites, but primarily in uninfected cells.

Next, we assessed the contribution of apoptosis to MHV-JHM-induced demyelinating disease by determining the quantity and location of apoptotic cells in the spinal cords of B6 mice with chronic demyelination. As discussed above, a variable percentage of suckling B6 mice inoculated intranasally with wild-type MHV-JHM 10 days after birth and protected from acute encephalitis by nursing with dams immunized to the virus later develop hindlimb paralysis associated with demyelination and virus persistence. TUNEL staining of spinal cords from these animals revealed significant quantities of apoptotic cells throughout the spinal cord (see below). Although TUNEL staining was present in both the grey and white mat-

TABLE 1. Virus titers in infected RAG1^{-/-} mice before and after adoptive transfer of immune splenocytes

Virus	Adoptive transfer?	No. of mice	Day p.i.	Titer (log ₁₀ PFU/g of tissue ± SE)
MHV-JHM ^a	No	6	31 ± 8	5.65 ± 0.45
J2.2-v1	No	2	5	2.93 ± 0.47
	No	5	10	4.66 ± 0.44
	No	3	15	4.87 ± 0.25
	Yes	3	10 ^b	3.61 ± 1.14

^a Inoculation in the presence of protective antibody.^b Seven days after transfer of 5×10^6 to 1×10^7 immune splenocytes.

ter, a majority of TUNEL-positive nuclei were seen in the white matter (Fig. 5A). Clusters of apoptotic cells were seen in the white matter and, by analogy to other reports (2), may comprise apoptotic lymphocytes. Using LFB-stained sections within 20 μ m of TUNEL-stained sections, we observed no obvious correlation between the quantity of apoptotic cells and the presence of demyelination, as similar numbers of TUNEL-positive nuclei were detected in areas with demyelination and

areas without demyelination (Fig. 5B). Double labeling for TUNEL-positive nuclei and virus antigen in spinal cords of these chronically infected immunocompetent mice revealed the presence of virus in areas containing TUNEL labeling with minimal cellular colocalization (data not shown).

The presence of TUNEL labeling does not depend on the presence of lymphocytes and is not sufficient to produce demyelination. Two possible sources of apoptotic cells following MHV-JHM infection are infiltrating immune cells and resident cells of the CNS. To determine the contribution of immune cells, spinal cords from RAG1^{-/-} mice infected with wild-type MHV-JHM or J2.2-v1 were examined for TUNEL labeling and viral antigen. RAG1^{-/-} mice infected with wild-type MHV-JHM were studied initially to facilitate direct comparison to immunocompetent B6 mice infected with the same virus. Extensive viral infection was detected throughout the gray and white matter in RAG1^{-/-} mice infected with wild-type MHV-JHM, although virtually no antigen-positive cells were double labeled with TUNEL. Among the infected cells detected in these mice were chains of cells in the white matter, with the morphology and distribution of intrafascicular oligodendrocytes. These cells did not demonstrate DNA fragmen-

FIG. 2. An increase in macrophages/microglia localize to areas of demyelination. Following the addition of lymphocytes on day 3 p.i., demyelination was detected 6 days after adoptive transfer (A, arrowheads). At this time, spinal cords of RAG1^{-/-} mice became densely packed with macrophages. A corresponding section stained by F4/80 immunohistochemistry demonstrated the overlap between macrophages/microglia and demyelinating lesions (B, arrowheads). F4/80 immunohistochemistry in spinal cords from RAG1^{-/-} mice infected with J2.2-v1 revealed a moderate number of macrophages. Fifteen days p.i., spinal cords from RAG1^{-/-} mice without demyelination contained macrophages/microglia with spiny morphology (C, arrowhead). In contrast, F4/80-positive cells found in demyelinating lesions following adoptive transfer exhibited a rounded appearance (D). Magnification in panels A and B = $\times 12.5$; magnification in panels C and D = $\times 50$.

FIG. 3. An increase in macrophages temporally correlates with demyelination. Quantification of F4/80-positive cells in RAG1^{-/-} mice infected with J2.2-v1 showed a rapid increase in numbers of macrophages/microglia throughout the spinal cord following adoptive transfer of splenocytes on day 3 p.i. The number of macrophages in spinal cords from adoptive transfer recipients with demyelination (at day 6 p.t., day 9 p.i.) was approximately three times the number seen in RAG1^{-/-} mice not receiving splenocytes and approximately seven times the number in mice 2 days p.t. (*, statistically significant difference between day 6 p.t. group and days 2 and 4 p.t. groups; $P < 0.005$ by Student's *t* test). Macrophage density was determined by counting the number of macrophages in the length of the spinal cord from sections approximately 1 mm apart, divided by the total area of the sections, as described in Materials and Methods. Three to five mice were analyzed for each group.

tation (Fig. 4C), and no chains of apoptotic cells were detected, suggesting that oligodendrocytes could be infected with MHV without evidence of apoptosis. Similar analyses of spinal cords from RAG1^{-/-} mice infected with J2.2-v1 revealed a comparable quantity and distribution of apoptotic cells (no demyelination evident [Fig. 5C and D]). TUNEL labeling was extensive throughout the spinal cord, with a majority of apoptotic cells being located in the white matter.

Following adoptive transfer of splenocytes into J2.2-v1-infected RAG1^{-/-} mice, the distribution of apoptotic cells was similar to that observed prior to adoptive transfer (demyelination evident [Fig. 5E and F]), although a statistically significant increase in the number of apoptotic cells was detected in these mice (59.50 ± 9.34 TUNEL-positive nuclei/100 μm^2 [mean $\pm$ standard error] versus 21.51 ± 6.20 for B6 mice infected chronically with wild-type MHV-JHM and 18.74 ± 4.44 for RAG1^{-/-} mice infected with J2.2-v1 [no demyelination]; $P < 0.05$ by Student's *t* test; at least five animals were analyzed for each group). As seen prior to adoptive transfer, TUNEL-positive nuclei were located both in gray and white matter, with a majority of labeling present in the white matter (Fig. 5F). Very few MHV-infected apoptotic cells were detected in these mice before or after transfer of splenocytes. These results demonstrate that the patterns of apoptosis in infected RAG1^{-/-} mice with and without demyelination and in B6 mice with chronic demyelination are similar, suggesting that apoptosis is not a key mediator of this process.

Astrocytes and macrophages undergoing apoptosis are detected following MHV-JHM infection of the CNS. Given that similar patterns of apoptosis were observed in the presence and absence of transferred cells, it seemed likely that a substantial fraction of TUNEL-positive cells were resident CNS

cells. To determine the identity of the cell type(s) undergoing apoptosis within the MHV-infected CNS, TUNEL staining in combination with immunohistochemistry for several cell-specific antigens was done. Antibody to GFAP was used to detect astrocytes, and F4/80 antibody was used to detect macrophages/microglia. Double labeling of TUNEL-stained spinal cord sections from symptomatic and asymptomatic RAG1^{-/-} mice, infected with either wild-type MHV-JHM or J2.2-v1, with immunohistochemistry for GFAP revealed the presence of very few apoptotic astrocytes (data not shown). However, astrocytes were frequently seen in close association with TUNEL-positive nuclei (Fig. 6A). In all infected mice, less than 1% of apoptotic cells were colabeled for TUNEL and GFAP. In contrast, immunohistochemistry for F4/80 in combination with TUNEL staining demonstrated a moderate number of apoptotic macrophages/microglia in both symptomatic and asymptomatic RAG1^{-/-} mice (Fig. 6B). Spinal cord sections from two MHV-JHM-infected B6 mice with hindlimb paralysis, three symptomatic J2.2-v1-infected RAG1^{-/-} mice, and three adoptive transfer recipients were quantitatively analyzed for the percentage of F4/80/TUNEL double-positive cells. In these animals, $7.9\% \pm 1.1\%$, $7.9\% \pm 1.6\%$, and $8.1\% \pm 2.0\%$, respectively, of TUNEL-positive cells were macrophages. In conclusion, colocalization of GFAP and F4/80 indicated that a few astrocytes and a moderate number of macrophages/microglia, respectively, were TUNEL positive.

DISCUSSION

RAG1^{-/-} mice infected with the attenuated MHV-JHM variant J2.2-v1 are a useful model for the dissection of mechanisms directly involved in virus-induced demyelination since demyelination is induced within a few days of adoptive transfer of immune splenocytes. This approach was originally described by Houtman and Fleming for infected SCID mice (14). In this study, we modified this system by the use of RAG1^{-/-} mice to minimize the capacity of the infected animal to develop any B- or T-cell-mediated immune response. In our initial experiments, this model was used to probe the role of macrophages and apoptosis in the development of demyelination.

In addition to demyelination, transfer of immune cells also resulted in rapid virus clearance from the gray matter. Although the mechanism of virus clearance from the gray matter is not clear, the absence of clearance from the white matter is also observed in other models of virus-induced demyelination (33). Clearance from gray matter may be in part cytokine mediated, since neuronal clearance is delayed in IFN- γ ^{-/-} mice infected with OBLV, an attenuated variant of MHV-JHM with tropism for olfactory neurons (24). Preferential persistence of virus in the white matter may result in continual immunologic activity, setting the stage for the destruction of myelin. There is not a strict relationship between virus clearance and demyelination in MHV-infected animals since demyelination occurs in the presence (normal B6 mice) and the absence ($\beta 2$ -microglobulin- and $\text{A}\beta$ -deficient mice) of virus clearance (14). Furthermore, the process of MHV clearance from glial cells may depend on cell-specific mechanisms. Parra et al. showed that IFN- γ may be necessary for MHV clearance from oligodendrocytes (37), while peroxin may be important for virus elimination from astrocytes and microglia (25).

Our results also suggest that apoptosis is not a major contributor to demyelination and in that sense are in general agreement with a previous report in which it was suggested that oligodendrocytes die by necrosis and not apoptosis (1). Also, Lin et al. suggested that apoptotic cells observed in the CNS of MHV-infected mice were related to the immune response

FIG. 4. MHV-JHM infection of the CNS leads to apoptosis. Extensive neuronal apoptosis was seen near the hippocampus in $RAG1^{-/-}$ mice with acute encephalitis. Double labeling of immunohistochemistry for virus antigen (red) and TUNEL (green) revealed the proximity of labeling without significant overlap (A), yet some double-positive cells were detected (B, arrow). Double labeling for virus antigen (red) and TUNEL (green) in a $RAG1^{-/-}$ mouse infected with wild-type MHV-JHM demonstrated the lack of infected cells undergoing apoptosis (C). Note the chain of infected cells with the morphology of intrafascicular oligodendrocytes (arrow). Alterations in nuclear morphology, including condensation of nuclear material (B, inset top, arrowhead) and budding of the membrane (B, inset bottom, arrowhead), confirmed the presence of apoptosis after MHV-JHM infection of the CNS. These morphological alterations were also observed in J2.2-v1-infected $RAG1^{-/-}$ mice (C, inset, arrowhead). DNA isolated from the olfactory bulb of B6 mice at day 4 p.i. was degraded into oligosomal fragments, characteristic of apoptosis (D). Lane 1, λ HindIII standard; lane 2, MHV-JHM-infected olfactory bulb DNA; lane 3, noninfected olfactory bulb DNA. Scale bar in panel A = 100 μ m; scale bar in panel B = 50 μ m; scale bar in panel B inset = 20 μ m.

since apoptotic cells were not detected in mice immunosuppressed by irradiation (25). Since apoptosis is observed in $RAG1^{-/-}$ mice in the absence of functional B and T cells, it is likely that at least some of the cells are CNS resident cells. Only F4/80-positive cells, representing either activated microglia or infiltrating macrophages/monocytes, were identified in significant numbers in the apoptotic population. Thus, the origin of the cells undergoing apoptosis remains uncertain. After adoptive transfer of immune splenocytes, the number of apoptotic cells is increased, but the distribution does not change. The additional apoptotic cells observed after splenocyte transfer are in large part, most likely, lymphocytes. In previous studies, apoptosis of antigen-specific and nonspecific T cells

within the CNS was clearly demonstrated (2). NK cells, which are normal in $RAG1^{-/-}$ mice, are also capable of undergoing apoptosis (36, 40) and may represent a fraction of TUNEL-positive cells both before and after adoptive transfer.

While we cannot determine with certainty whether some of these additional apoptotic cells are oligodendrocytes, we think that it is more likely that oligodendrocytes die by necrosis. Prior to adoptive transfer, infected intrafascicular oligodendrocytes are readily detected in $RAG1^{-/-}$ mice, without any evidence for similar arrays of apoptotic cells. Transfer of immune cells results in virus clearance and probably destruction of infected oligodendrocytes. As in the untreated infected $RAG1^{-/-}$ mice, no evidence for infected apoptotic oligoden-

FIG. 5. Patterns of apoptosis are similar in spinal cords from mice with and without demyelination. (A and B) B6 mice infected with wild-type MHV-JHM. TUNEL-labeled cells were observed throughout the white matter predominantly, with some apoptotic cells present in the gray matter of the spinal cord (A, tissue border signified by +). This mouse was infected with wild-type MHV-JHM at 10 days of age and nursed by a dam immunized to MHV-JHM. It developed hindlimb paralysis at day 36 p.i. Areas containing extensive demyelination (B, black arrowhead) as well as minimally disturbed areas of white matter (B, white arrowhead) both contained TUNEL-positive nuclei. Images from panels A and B are from adjacent sections. (C to F) RAG1^{-/-} mice infected with J2.2-v1. These mice displayed very similar patterns of TUNEL staining in the spinal cord, with the majority of labeling detected in the white matter (C, tissue border demarcated by +). Demyelination was not detected in these mice (D). Seven days after the adoptive transfer of spleen cells, a qualitatively similar pattern of TUNEL staining was distributed mostly throughout the white matter of the spinal cord (E). This adoptive transfer of splenocytes resulted in demyelination without a change in the distribution of apoptotic cells (F, arrows). Scale bar (applies to all panels) = 200 μ m.

drocytes was detected, although these observations are complicated by disruption of normal white matter architecture caused by the infiltrating lymphocytes. We have been unable to identify an oligodendrocyte-specific antibody that could be used in conjunction with the TUNEL assay and thus are unable to verify this conclusion by another approach. Our results are

generally consistent with those of Barac-Latas et al., who described a complex sequence of overlapping patterns of pathology involving oligodendrocyte necrosis with a lesser component of apoptosis in MHV-infected Lewis rats (1).

In all the experimental animals analyzed in this work, approximately 10% of the apoptotic cells were macrophages and

FIG. 6. TUNEL and immunohistochemical colabeling. TUNEL staining (green) in combination with anti-GFAP immunohistochemistry (red) revealed astrocytes near TUNEL-positive nuclei in the spinal cord of RAG1^{-/-} mice infected with J2.2-v1 (A). A greater fraction of the apoptotic cells were macrophages, as identified by colabeling (arrow) of F4/80 immunohistochemistry (brown) with TUNEL (green) (B). Scale bar (applies to both panels) = 50 μ m.

the rest were unidentified. In other neuropathological settings, apoptosis of macrophages/microglia has been commonly observed (13). In general, however, it has been difficult to identify which types of cells are undergoing apoptosis, since specific markers are lost as proteolysis occurs during apoptotic death (27). The majority of the apoptotic cells were uninfected but in close proximity to regions with intense virus replication, suggesting that apoptosis was triggered by a soluble factor. This factor could be produced by uninfected or infected resident CNS cells or NK cells or by CD4⁺ CD8⁺ lymphocytes present in RAG1^{-/-} mice (8, 30). Immunoregulatory molecules such as nitric oxide synthase 2 and tumor necrosis factor alpha, known to be involved in the induction of apoptosis (31, 32), are both elevated in the CNS of B6 mice infected with MHV (12, 45) and synthesized largely by uninfected astrocytes located adjacent to areas of MHV replication and demyelination (45). Previous results suggest that nitric oxide, but not tumor necrosis factor alpha, may be involved in MHV-induced demyelination (23, 43).

While our results suggest that apoptosis is not a major factor in the development of demyelination, adoptive transfer of splenocytes induced demyelination and the appearance of large numbers of activated macrophages. Our results suggest that the demyelinating process has actually begun in RAG1^{-/-} mice since a small number of lipid-laden macrophages can be detected in RAG1^{-/-} mice prior to adoptive transfer (data not shown). Thus, the transferred cells or their secreted products may be required for propagating, but not initiating, demyelination.

The presence of large numbers of lipid-laden macrophages is readily detected in the CNS of rodents with experimentally induced demyelination and of humans with MS. The importance of macrophages in the demyelinating process is emphasized by results described in several recent studies. In rodents with EAE or demyelination induced by TMEV infection, treatment with drugs which deplete all hematogenously derived macrophages (such as liposome-encapsulated dichloromethylene diphosphonate) greatly decreases the amount of demyelination (16, 46). In the case of EAE, these treatments appear to interfere with antigen presentation and/or T-cell activation. T cells cross the blood-brain barrier and, in the case of Lewis rats, are able to enter the parenchyma. However, in the ab-

sence of macrophages, they are unable to initiate the process of demyelination (16). In mice chronically infected with TMEV, infiltrating macrophages are a major reservoir for the virus (26). Depletion of these cells greatly decreases virus burden and, concomitantly, demyelination. Of note, depletion of hematogenous macrophages in MHV-infected mice does not prevent the appearance of macrophages at sites of virus replication or inhibit demyelination (50). These results suggest that in MHV-infected animals, antigen load is sufficiently great so that blood-borne macrophages are not required for activation of T cells or penetration into the CNS parenchyma.

The results presented herein show that macrophages/microglia in the absence of lymphocytes, in reciprocal fashion to Lewis rats prone to developing EAE, are able to enter the parenchyma and migrate to sites of virus replication but not cause demyelination. Several chemokines known to be involved in macrophage recruitment and activation, including MIP-1 β , CRG-2, MIP-2, MCP-1, MCP-3, and RANTES, are upregulated in the CNS of MHV-infected mice (21). With the exception of RANTES, all of these are secreted primarily by astrocytes. One possibility is that T lymphocytes are required to stimulate the production of these chemokines by resident glial cells. This in turn would lead to additional recruitment and activation of microglia/macrophages and thereby propagate the demyelinating cascade. The model system of RAG1^{-/-} mice infected with the attenuated J2.2-v1 mutant will be useful for dissecting the steps important in macrophage recruitment and in the development of MHV-induced demyelination.

ACKNOWLEDGMENTS

We thank M. Dailey, T. Lane, C. M. Stoltzfus, and J. Fleming for critically reviewing the manuscript.

This research was supported in part by grants from the National Institutes of Health (NS 36592) and the National Multiple Sclerosis Society (RG2864-A-2). G.F.W. was also supported by NRSA predoctoral fellowship MH12066-02.

REFERENCES

1. Barac-Latas, V., G. Suchanek, H. Breitschopf, A. Stuehler, H. Wege, and H. Lassmann. 1997. Patterns of oligodendrocyte pathology in coronavirus-induced subacute demyelinating encephalomyelitis in the Lewis rat. *Glia* 19: 1-12.

2. Bauer, J., M. Bradl, W. F. Hickey, S. Forss-Petter, H. Breitschopf, C. Linington, H. Wekerle, and H. Lassmann. 1998. T-cell apoptosis in inflammatory brain lesions: destruction of T cells does not depend on antigen recognition. *Am. J. Pathol.* **153**:715–724.
3. Bonetti, B., and C. S. Raine. 1997. Multiple sclerosis: oligodendrocytes display cell death-related molecules in situ but do not undergo apoptosis. *Ann. Neurol.* **42**:74–84.
4. Cuzner, M. L. 1997. Microglia in health and disease. *Biochem. Soc. Trans.* **25**:671–673.
5. Diemel, L. T., C. A. Copelman, and M. L. Cuzner. 1998. Macrophages in CNS remyelination: friend or foe? *Neurochem. Res.* **23**:341–347.
6. Dowling, P., W. Husar, J. Menonna, H. Donnenfeld, S. Cook, and M. Sidhu. 1997. Cell death and birth in multiple sclerosis brain. *J. Neurol. Sci.* **149**:1–11.
7. D'Souza, S. D., B. Bonetti, V. Balasingam, N. R. Cashman, P. A. Barker, A. B. Troutt, C. S. Raine, and J. P. Antel. 1996. Multiple sclerosis: Fas signaling in oligodendrocyte cell death. *J. Exp. Med.* **184**:2361–2370.
8. Falk, I., A. J. Potocnik, T. Barthlott, C. N. Levell, and K. Eichmann. 1996. Immature T cells in peripheral lymphoid organs of recombinase-activating gene-1/2-deficient mice. *J. Immunol.* **156**:1362–1368.
9. Fleming, J. O., M. D. Trousdale, F. A. El-Zaatari, S. A. Stohlman, and L. P. Weiner. 1986. Pathogenicity of antigenic variants of murine coronavirus JHM selected with monoclonal antibodies. *J. Virol.* **58**:869–875.
10. Fleming, J. O., F.-I. Wang, M. D. Trousdale, D. R. Hinton, and S. A. Stohlman. 1993. Interaction of immune and central nervous systems: contribution of anti-viral Thy-1+ cells to demyelination induced by coronavirus JHM. *Reg. Immunol.* **5**:37–43.
11. Gold, R., H.-P. Hartung, and H. Lassmann. 1997. T-cell apoptosis in autoimmune diseases: termination of inflammation in the nervous system and other sites with specialized immune-defense mechanisms. *Trends Neurosci.* **20**:399–404.
12. Grzybicki, D. M., K. Kwak, S. Perlman, and S. P. Murphy. 1997. Nitric oxide synthase type II expression by different cell types in MHV-JHM encephalitis suggests distinct roles for nitric oxide in acute versus persistent virus infection. *J. Neuroimmunol.* **73**:15–27.
13. Hajimohamadreza, I., and J. M. Treherne. 1997. The role of apoptosis in neurodegenerative diseases. *Prog. Drug Res.* **48**:55–98.
14. Houtman, J. J., and J. O. Fleming. 1996. Dissociation of demyelination and viral clearance in congenitally immunodeficient mice infected with murine coronavirus JHM. *J. Neurovirol.* **2**:101–110.
15. Houtman, J. J., and J. O. Fleming. 1996. Pathogenesis of mouse hepatitis virus-induced demyelination. *J. Neurovirol.* **2**:361–376.
16. Huitinga, L., N. Van Rooijen, C. J. De Groot, and C. D. Dijkstra. 1990. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. *J. Exp. Med.* **172**:1025–1033.
17. Kerr, J. R. F., A. H. Wyllie, and A. R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer.* **26**:239–257.
18. Kruisbeek, A. M. 1991. Current protocols in immunology, vol. 1. John Wiley & Sons, New York, N.Y.
19. Kyuwa, S., and S. A. Stohlman. 1990. Pathogenesis of a neurotropic murine coronavirus, strain JHM in the central nervous system of mice. *Semin. Virol.* **1**:273–280.
20. Lampert, P. W., J. K. Sims, and A. J. Kniazeff. 1973. Mechanisms of demyelination in JHM virus encephalomyelitis. *Acta Neuropathol.* **24**:76–85.
21. Lane, T. E., V. C. Asensio, N. Yu, Paoletti, A. D., I. L. Campbell, and M. J. Buchmeier. 1998. Dynamic regulation of alpha- and beta-chemokine expression in the central nervous system during mouse hepatitis virus-induced demyelinating disease. *J. Immunol.* **160**:970–978.
22. Lane, T. E., and M. J. Buchmeier. 1997. Murine coronavirus infection: a paradigm for virus-induced demyelinating disease. *Trends Microbiol.* **5**:9–14.
23. Lane, T. E., H. S. Fox, and M. J. Buchmeier. 1998. Inhibition of nitric oxide synthase-2 reduces the severity of mouse hepatitis virus-induced demyelination: implication for NOS2/NO regulation of chemokine expression and inflammation. *J. Neurovirol.* **5**:48–54.
24. Lane, T. E., A. D. Paoletti, and M. J. Buchmeier. 1997. Disassociation between the in vitro and in vivo effects of nitric oxide on a neurotropic murine coronavirus. *J. Virol.* **71**:2202–2210.
25. Lin, M. T., S. A. Stohlman, and D. R. Hinton. 1997. Mouse hepatitis virus is cleared from the central nervous system of mice lacking perforin-mediated cytotoxicity. *J. Virol.* **71**:383–391.
26. Lipton, H. L., G. Twaddle, and M. L. Jelachich. 1995. The predominant virus antigen burden is present in macrophages in Theiler's murine encephalitis virus-induced demyelinating disease. *J. Virol.* **69**:2525–2533.
27. MacDonald, R. G., T. P. Martin, and J. A. Cidlowski. 1980. Glucocorticoids stimulate protein degradation in lymphocytes: a possible mechanism of steroid-induced cell death. *Endocrinology* **107**:1512–1524.
28. McKnight, A. J., A. J. Macfarlane, P. Dri, L. Turely, and A. C. Willis. 1996. Molecular cloning of F4/80, a murine macrophage-restricted cell surface glycoprotein with homology to the G protein-linked transmembrane 7 hormone receptor family. *J. Biol. Chem.* **271**:486–489.
29. Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**:869–877.
30. Mombaerts, P., E. Mizoguchi, H.-G. Ljunggren, J. Iacomini, H. Ishikawa, L. Wang, M. J. Grusby, L. H. Glimcher, H. J. Winn, A. K. Bhan, and S. Tonegawa. 1994. Peripheral lymphoid development and function in TCR mutant mice. *Int. Immunol.* **6**:1061–1070.
31. Monasta, G., A. Cabrelle, A. Zamboni, A. Rosato, B. Macino, D. Collavo, and P. Zanovello. 1996. Membrane form of TNF alpha induces both cell lysis and apoptosis in susceptible target cells. *Cell. Immunol.* **171**:102–110.
32. Nicotera, P., E. Bonfoco, and B. Brune. 1995. Mechanisms for nitric oxide-induced cell death: involvement of apoptosis. *Adv. Neuroimmunol.* **5**:411–420.
33. Njenga, M. K., K. Asakura, H. S. F., P. Wettstein, L. R. Pease, and M. Rodriguez. 1997. The immune system preferentially clears Theiler's virus from the gray matter of the central nervous system. *J. Virol.* **71**:8592–8601.
34. Nonoyama, S., and H. D. Ochs. 1996. Immune deficiency in SCID mice. *Int. Rev. Immunol.* **13**:289–300.
35. Oberhaus, S. M., R. L. Smith, G. H. Clayton, T. S. Dermody, and K. L. Tyler. 1997. Reovirus infection and tissue injury in the mouse central nervous system are associated with apoptosis. *J. Virol.* **71**:2100–2106.
36. Ortaldo, J. R., A. T. Mason, and J. J. O'Shea. 1995. Receptor-induced death in human natural killer cells: involvement of CD16. *J. Exp. Med.* **181**:339–344.
37. Parra, B., D. R. Hinton, N. W. Marten, C. C. Bergmann, M. T. Lin, C. S. Yang, and S. A. Stohlman. 1999. IFN-gamma is required for viral clearance from central nervous system oligodendroglia. *J. Immunol.* **162**:1641–1647.
38. Perlman, S., R. Schelper, E. Bolger, and D. Ries. 1987. Late onset, symptomatic, demyelinating encephalomyelitis in mice infected with MHV-JHM in the presence of maternal antibody. *Microb. Pathog.* **2**:185–194.
39. Ross, C. P., M. Delcroix, I. Huitinga, A. McAllister, J. van Rooijen, E. Claassen, and M. Brahic. 1997. Role of macrophages during Theiler's virus infection. *J. Virol.* **71**:3336–3340.
40. Ross, M. E., and M. A. Caligiuri. 1997. Cytokine-induced apoptosis of human natural killer cells identifies a novel mechanism to regulate the innate immune response. *Blood* **89**:910–918.
41. Sabelko, K. A., K. A. Kelly, M. H. Nahm, A. H. Cross, and J. H. Russell. 1997. Fas and fas ligand enhance the pathogenesis of experimental allergic encephalitis, but are not essential for immune privilege in the central nervous system. *J. Immunol.* **159**:3096–3099.
42. Sriram, S., and M. Rodriguez. 1997. Indictment of the microglia as the villain in multiple sclerosis. *Neurology* **48**:464–470.
43. Stohlman, S. A., D. R. Hinton, D. Cua, E. Dimacali, J. Sensintaffar, F. M. Hoffman, S. M. Tahara, and Q. Yao. 1995. Tumor necrosis factor expression during mouse hepatitis virus-induced demyelinating encephalomyelitis. *J. Virol.* **69**:5898–5903.
44. Stohlman, S. A., and L. P. Weiner. 1981. Chronic central nervous system demyelination in mice after JHM virus infection. *Neurology* **31**:38–44.
45. Sun, N., D. Grzybicki, R. F. Castro, S. P. Murphy, and S. Perlman. 1995. Activation of astrocytes in the spinal cord of mice chronically infected with a neurotropic coronavirus. *Virology* **213**:482–493.
46. Tran, E. H., K. Hoekstra, N. Van Rooijen, C. D. Dijkstra, and T. Owens. 1998. Immune invasion of the central nervous system parenchyma and experimental allergic encephalomyelitis, but not leukocyte extravasation from blood, are prevented in macrophage-depleted mice. *J. Immunol.* **161**:3767–3775.
47. Waldner, H., R. A. Sobel, E. Howard, and V. K. Kuchroo. 1997. Fas- and FasL-deficient mice are resistant to induction of autoimmune encephalomyelitis. *J. Immunol.* **159**:3100–3103.
48. Wang, F.-I., D. R. Hinton, W. Gilmore, M. D. Trousdale, and J. O. Fleming. 1992. Sequential infection of glial cells by the murine hepatitis virus JHM strain (MHV-4) leads to a characteristic distribution of demyelination. *Lab. Invest.* **66**:744–754.
49. Wang, F.-I., S. A. Stohlman, and J. O. Fleming. 1990. Demyelination induced by murine hepatitis virus JHM strain (MHV-4) is immunologically mediated. *J. Neuroimmunol.* **30**:31–41.
50. Xue, S., N. Sun, N. van Rooijen, and S. Perlman. 1999. Depletion of blood-borne macrophages does not reduce demyelination in mice infected with a neurotropic coronavirus. *J. Virol.* **73**:6327–6334.