Depletion of Blood-Borne Macrophages Does Not Reduce Demyelination in Mice Infected with a Neurotropic Coronavirus

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Mice infected with the neurotropic coronavirus mouse hepatitis virus strain JHM (MHV-JHM) develop a chronic demyelinating disease with symptoms of hindlimb paralysis. Histological examination of the brains and spinal cords of these animals reveals the presence of large numbers of activated macrophages/microglia. In two other experimental models of demyelination, experimental allergic encephalomyelitis and Theiler's murine encephalomyelitis virus-induced demyelination, depletion of hematogenous macrophages abrogates the demyelinating process. In both of these diseases, early events in the demyelinating process are inhibited by macrophage depletion. From these studies, it was not possible to determine whether infiltrating macrophages were required for late steps in the process, such as myelin removal. In this study, we show that when macrophages are depleted with either unmodified or mannosylated liposomes encapsulating dichloromethylene diphosphate, the amount of demyelination detected in MHV-infected mice is not affected. At a time when these cells were completely depleted from the liver, approximately equivalent numbers of macrophages were present in the spinal cords of control and drug-treated animals. These results suggest that blood-borne macrophages are not required for MHV-induced demyelination and also suggest that other cells, such as perivascular macrophages or microglia, perform the function of these cells in the presence of drug.

Mouse hepatitis virus strain JHM (MHV-JHM) causes acute and chronic neurological diseases in susceptible strains of rodents. The acute encephalitis is characterized by extensive infection of neurons and is a fatal disease. In some experimental models, mice are protected from the acute, fatal disease either by infection with attenuated virus or by passive infusions of antiviral antibodies or T cells. Under these conditions, this virus also causes acute and chronic inflammatory demyelinating diseases. These diseases serve as a model for the human demyelinating disease multiple sclerosis (reviewed in references 8 and 32).

Like other experimental demyelinating diseases, such as experimental allergic encephalomyelitis (EAE) and the demyelinating encephalomyelitis caused by Theiler's murine encephalomyelitis virus (TMEV), the pathogenesis of MHV-JHM-induced demyelination is in large part immune system mediated (7, 39). Demyelination does not occur in mice with severe combined immunodeficiency (SCID mice) or in *RAG-1*^{-/-} mice but is present in SCID mice reconstituted with T cells after viral infection (reference 7 and unpublished observations). Another feature of MHV-JHM-induced demyelination is the presence of large numbers of activated macrophages/microglia (13, 21, 29, 33). These cells are likely to participate in antigen presentation and in myelin removal.

Extensive infiltration of macrophages/microglia is also detected in the central nervous system (CNS) of humans with multiple sclerosis and in rodents with either EAE or TMEV-induced demyelination (15, 24–26). In both of the latter experimental diseases, pharmacological inhibition of macrophage function with mannosylated liposomes encapsulating dichlo-

romethylene diphosphonate (Cl₂MDP) or with *N,N'*-bis{3,5-bis[1-[(aminoiminomethyl)hydrazono]ethyl]phenyl} decanediamide tetrahydrochloride (CNI-1493) prevents or ameliorates the development of demyelination (1, 10, 11, 17, 26, 35). These agents eliminate or inhibit the function of blood-borne macrophages but have much less or no effect on the function of tissue macrophages such as microglia or of dendritic cells (1, 3, 38). This is most probably because these cells are not accessible to the drug (36). The results obtained from mice with EAE suggest that blood-borne macrophages are important in the initiation and/or propagation of the immune response. Of note, while Cl₂MDP encapsulated by unmodified liposomes (Cl₂MDP-L) or by mannosylated liposomes (Cl₂MDP-mnL) caused depletion of macrophages from the spleen and liver, only Cl₂MDP-mnL prevented demyelination (11). The basis of this differential effect is not known at present (38). In mice chronically infected with TMEV, macrophages are the predominant reservoir for the virus and elimination of these cells results in a great decrease in the amount of virus present in the infected CNS (15, 26).

In one model of MHV-JHM-induced demyelination, suckling C57BL/6 (B6) mice are infected intranasally with MHV and are nursed by dams previously immunized with live MHV-JHM (21). Although these mice do not develop acute encephalitis, a variable fraction (40 to 90%) later develop hindlimb paralysis with histological evidence of inflammatory demyelination 3 to 8 weeks after infection. Infectious virus can be isolated from the CNS of mice with chronic demyelination. We showed previously that in every case this virus is mutated in the immunodominant CD8 T-cell epitope encompassing residues 510 to 518 of the surface (S) glycoprotein (22). We also showed that infection of suckling B6 mice with mutated virus resulted in increased morbidity and mortality compared to infection with wild-type virus, with most of the animals having clinical disease by 21 days postinoculation (p.i.) (23). Widespread de-

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myelination accompanied by extensive inflammatory infiltrates is present in the CNS of these mice.

Macrophages are likely to be the final effectors in MHV-induced demyelination. The high levels of viral antigen and the exuberance of the immune response raised the possibility that several types of cells would be involved in initiating and propagating the immune response and that hematogenous macrophages would not play as critical a role in these processes as in EAE. In addition, we show that macrophages are not the primary reservoir for virus in mice chronically infected with MHV, unlike for TMEV-infected animals (15). Therefore, depletion of macrophages would not decrease the virus load appreciably. However, hematogenous macrophages could still play a critical role as the final effectors of MHV-induced demyelination, i.e., involved in stripping myelin from axons. In this report, we show that blood-borne macrophages are not essential for virus-induced demyelination since demyelination occurs to the same extent in the presence and absence of these cells.

MATERIALS AND METHODS

Viruses. Wild-type MHV-JHM, originally obtained from S. Weiss, University of Pennsylvania, was grown and subjected to titer determination on BALB/c-derived 17CL-1 cells as previously described (21). Cytotoxic T lymphocyte (CTL) escape mutants were previously isolated from mice with chronic demyelination and propagated in 17CL-1 cells (22, 23). The variant used in this study was mutated at nucleotide 1541 (A to G) in the S gene, resulting in an amino acid change in the anchor residue for binding to the H-2D^b molecule (asparagine to serine).

Animals. MHV-negative 6-week-old B6 mice were purchased from the National Cancer Institute (Frederick, Maryland). To obtain mice with chronic demyelination, suckling B6 mice were inoculated intranasally with either wild-type or CTL escape mutant virus (2.5×10^4 PFU) at 10 days of age and were nursed by dams previously immunized with live MHV-JHM (21). Mice infected with wild-type virus develop hindlimb paralysis between 3 and 8 weeks p.i., whereas most mice infected with mutated virus are symptomatic between 15 and 22 days p.i. (23). Symptomatic animals were euthanized with an overdose of Nembutal and perfused with phosphate-buffered saline (PBS) via the left ventricle. Livers, spleens, brains, and spinal cords were harvested from these mice.

Tissue processing. For immunohistochemistry, samples were fixed with Histocchoice (Amresco, Solon, Ohio) for 48 h and then embedded in paraffin. For double-immunofluorescence assays, samples were frozen in Tissue-Tek II O.C.T. medium (Miles Laboratory, Elkhart, Ind.) in acetone-dry ice and stored at -70°C before being sectioned.

Viral titers from infected CNS tissue. To determine viral titers, tissue was homogenized and the supernatants were subjected to titer determination on 17CL-1 cells as described previously (21).

Preparation of liposomes. Unmodified and mannoseylated liposomes were constructed from phosphatidylcholine, cholesterol, and mannose as previously described (38). Briefly, 86 mg of phosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany) and 8 mg of cholesterol (Sigma, St. Louis, Mo.) at a molar ratio of 6:1 were dissolved in chloroform in a round-bottom flask. For the synthesis of mannoseylated liposomes, 70.9 mg of phosphatidylcholine and 10.8 mg of cholesterol were dissolved in chloroform and added to 3.6 mg of *p*-amino-phenyl- α -D-mannopyranoside. The thin film formed on the interior of the flask after low-vacuum rotary evaporation at 37°C was dispersed in 10 ml of PBS containing 1.89 g of Cl₂MDP (a kind gift of Boehringer GmbH, Mannheim, Germany) by gentle rotation for 10 min. Free Cl₂MDP was removed by rinsing the liposomes with sterile PBS and centrifuging them for 30 min at $25,000 \times g$ at 16°C . Finally, the liposomes were resuspended in 4 ml of PBS. For depletion of blood-borne macrophages, 0.1 ml of the suspension was injected intraperitoneally per 10 g of body weight.

Experimental paradigm. The effect of Cl₂MDP-L and Cl₂MDP-mnL on MHV-induced demyelination was determined in two separate sets of experiments. In the first set of experiments, 14 suckling mice were treated with Cl₂MDP-L 1 day prior to infection with MHV and every 5 days thereafter. Ten littermates of the treated mice did not receive the drug and served as controls. In the second set of experiments, 11 suckling mice were treated with Cl₂MDP-mnL 1 day prior to MHV infection and every 5 days thereafter. Six littermates of the treated animals served as controls. In both experiments, the suckling mice were nursed by dams immunized to MHV and the data obtained were analyzed as described above and in Results.

Antibodies. Two antibodies (Ab) were used to detect macrophages. Rat anti-macrophage monoclonal antibody (MAb) F4/80 (CI:A3-1; Serotec, Oxford, England) recognizes a protein with homology to a family of hormone receptors (18). Rat anti-Mac-1 (clone M1/70) recognizes iC3b and was obtained from M. Dailey,

University of Iowa. Murine Ab recognizing major histocompatibility complex (MHC) class I antigen (anti-H-2K^b/H-2D^b [clone 20-8-4]) and MHC class II antigen (anti-I-A^b [clone 25-9-17S]) were provided by M. Dailey. To detect astrocytes, a murine Ab recognizing glial fibrillary acidic protein (anti-GFAP [clone G-A-5]) was purchased from Sigma Immuno Chemicals. Murine Ab recognizing the S (MAb 5A13.5 and 4B19.2) and nucleocapsid (N) (MAb 5B188.2) proteins were provided by M. Buchmeier, The Scripps Research Institute. Biotinylated goat anti-rat immunoglobulin G and biotinylated goat anti-mouse immunoglobulin G Ab were purchased from Vector Laboratories (Burlingame, Calif.).

Immunohistochemistry. Paraffin-embedded sections were cut on a microtome at thicknesses of 8 to 12 μm and mounted on precleaned Superfrost/Plus Microscope Slides (Fisher Scientific, Pittsburgh, Pa.). The sections were dewaxed with xylene, rehydrated, and blocked with CAS BLOCK (Zymed Laboratories, South San Francisco, Calif.). After removal of the blocking solution, sections were incubated with F4/80 antibody (1:50 dilution) at 4°C overnight. After being washed, the sections were incubated with biotinylated secondary Ab (1:500 dilution) for 1 h at room temperature. Antigen-Ab complex were visualized with peroxidase-conjugated avidin (Jackson ImmunoResearch Laboratories, West Grove, Pa.) (1:1,000 dilution) with 3,3'-diaminobenzidine (Sigma) as the final substrate. As negative controls, sections from the same spinal cord were processed in the absence of primary Ab.

Double-immunofluorescence assays. To determine whether macrophages/microglia expressed MHC class I and class II antigens, cells were dually labeled with anti-Mac-1 Ab and either MHC class I or class II Ab by previously described methods (34). Briefly, 25- to 30- μm -thick frozen sections of spinal cord were cut with a cryostat and mounted on silane-treated slides. The sections were then fixed in 2.5 to 4% paraformaldehyde in phosphate-lysine-periodate buffer (19) at 4°C for 30 min, washed with PBS, and incubated with 5% normal goat or rabbit serum. The sections were then incubated with the primary Ab anti-Mac-1 Ab (1:50 to 1:100) and anti-I-A^b Ab (1:200) or anti-H-2K^b/H-2D^b Ab (1:200) at 4°C overnight or at room temperature for 2 h. After being washed, samples were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse Ab (1:200) and Texas Red-conjugated goat anti-rat Ab (1:200) for 1 to 2 h at room temperature. The slides were mounted with Vectashield medium (Vector Laboratories) and examined and photographed with an Olympus BH-2 microscope with epifluorescence light excitation. No evidence of spillover between the two fluorescent tags was observed in control experiments. As negative controls, sections from the same spinal cord were processed in the absence of primary Ab. No labeling was observed in these sections.

Double-labeling immunocytochemistry and in situ hybridization. In situ hybridization with an antisense riboprobe for MHV RNA was combined with immunohistochemistry for F4/80 or Mac-1. Briefly, frozen sections from the spinal cords of mice were prepared, fixed in 2.5 to 4% phosphate-lysine-periodate buffer, and processed for immunohistochemistry as described above. In situ hybridization was then performed as previously described (34). The samples were then dipped in NTB-2 emulsion (Eastman Kodak, Rochester, N.Y.) for 2 weeks. The slides were stained with cresyl violet and examined by bright-field and dark-field light microscopy. Control experiments included omitting the primary Ab or using an irrelevant Ab instead of a specific Ab.

Histology. Paraffin embedded sections were cut, mounted on slides, dewaxed, and stained with luxol fast blue (LFB) to detect areas of demyelination.

Quantitative study of demyelination by image analysis. Sequential 8- to 12- μm -thick sagittal sections from spinal cords were cut. A total of 14 to 26 slides with four to seven sections per slide were prepared for each spinal cord. All the sections were stained with LFB. Three to five sections from each animal were imaged by using light microscopy and a video camera at a magnification of $\times 200$. Approximately 12 to 45 images were required to encompass an entire sagittal section. An image analysis program, Vtrace (Image Analysis Facility, University of Iowa), was used to delineate myelinated and demyelinated areas. The marked areas were digitized, and two calculations were performed with these data. First, the total demyelinated area on all of the sections was determined and divided by the sum of the demyelinated and myelinated areas. Alternatively, this fraction was determined for each section and then the average of these values was calculated for each mouse. Similar results were obtained by the two methods.

Statistical analysis. The statistical significance was determined as described in the figure legends. Analysis was performed with the help of the Biostatistics Core Facility at the University of Iowa.

RESULTS

Macrophages/microglia in the CNS of mice with chronic demyelination express MHC class I and class II antigens. Infiltrating blood-borne macrophages and activated resident microglia are abundant in the CNS of mice with acute encephalitis or chronic demyelination induced by MHV-JHM (13, 21, 33) (Fig. 1D). Our previous results suggested, based on morphological criteria, that only macrophages expressed MHC class I and class II molecules in the CNS of mice with chronic

FIG. 1. Treatment with $\text{Cl}_2\text{MDP-L}$ or $\text{Cl}_2\text{MDP-mnL}$ 1 day prior to infection results in depletion of F4/80 staining from the liver but not the CNS and has no effect on demyelination. Control (A, D, and G), $\text{Cl}_2\text{MDP-L}$ -treated (B, E, and H), and $\text{Cl}_2\text{MDP-mnL}$ -treated (C, F, and I) mice were harvested at 21 days p.i. Livers (A to C) and spinal cords (D to I) were either assayed for the presence of macrophages/microglia with F4/80 antibody (A to F) or stained for myelin (G to I) as described in Materials and Methods. Control samples were harvested from the experiment in Fig. 3 and Table 1. Bar, 100 μm (A to C), 50 μm (D to F), and 200 μm (G to I).

demyelination (34). Since the morphology of astrocytes can be quite variable, particularly in mice with pathological conditions, cells were simultaneously monitored for the expression of macrophage/microglia-specific antigen or astrocyte-specific antigen and for that of MHC class I or class II antigens. As shown in Fig. 2, the overlap between cells expressing Mac-1 and MHC class I and class II antigens was nearly complete, suggesting that cells of the macrophage lineage expressed MHC antigens and that these molecules were present only on Mac-1^+ cells. In contrast, no cells positive for both glial fibrillary acidic protein, a protein found in the CNS only in astrocytes, and MHC class I and II antigens could be detected (data not shown). These observations confirm our preliminary results and suggest that if MHC antigen is expressed by astrocytes in mice with the chronic demyelinating disease, it must be

at significantly lower levels than can be detected on macrophages/microglia. They also show that macrophages/microglia are activated in the CNS of mice persistently infected with MHV-JHM since MHC class I and II antigens are expressed only at low levels in the uninfected CNS (14).

Depletion of macrophages with $\text{Cl}_2\text{MDP-L}$ at 7 days p.i. does not affect the extent of demyelination. As described above, treatment with liposomes containing Cl_2MDP , by selectively depleting hematogenous macrophages, abrogated the development of demyelination in two experimental models of demyelination (EAE and chronic infection with TMEV). This drug was used in the present study because its effects have been extensively analyzed and it is known to have minimal toxicity after long-term administration (36, 38). The exact mechanism of action of this drug within macrophages is not known, but it

FIG. 2. Macrophages/microglia express MHC class I and II molecules in the CNS of mice with chronic demyelination. The spinal cord of an animal harvested 25 days p.i. was assayed by a double-immunofluorescence assay for both Mac-1 (A and C) and MHC class I (B) or II (D) antigen as described in Materials and Methods. Nearly every Mac-1-positive cell also expressed MHC class I and class II antigen. Arrows in panels A and B and arrowheads in panels C and D indicate examples of doubly labeled cells. Bar, 100 μ m.

may deplete intracellular iron stores or directly affect ATP metabolism. To determine if this reagent or its unmodified form ($\text{Cl}_2\text{MDP-L}$) had a similar effect on MHV-infected mice, animals were treated initially at 7 days p.i. and every 5 days thereafter. This regimen results in nearly completely depletion of macrophages from the liver and specific subsets of the spleen within 24 h of treatment (37) and maintains this depletion for the duration of the experiment (36). In these experiments, the mice were infected with a virus mutated in the immunodominant CTL epitope (S-510-518) since infection with the CTL escape mutant results in the development of demyelination in a higher percentage of mice than does infection with wild-type virus (23). The ability to cause disease in a higher percentage of recipients was particularly useful in this study, in which the effect of treatment with a drug was evaluated. Drug was delivered by i.p. inoculation because i.v. inoculation was technically difficult in these young mice. Previous reports suggested that i.v. and i.p. administrations of Cl_2MDP were equally efficacious (38). In these initial experiments, the drug was delivered at 7 days p.i. because treatment at this time blocked demyelination in TMEV-infected mice (26). The mice were euthanized at 21 days p.i. or earlier (15 to 18 days) if moribund. Our initial experiments showed that treatment with $\text{Cl}_2\text{MDP-L}$ beginning at 7 days p.i. did not decrease macrophage/microglia infiltration into the CNS and had no effect on the development of demyelination (data not shown).

Depletion of macrophages with $\text{Cl}_2\text{MDP-L}$ 1 day prior to virus inoculation does not decrease the amount of demyelination. The most stringent way to ensure that macrophages are not activated before treatment with Cl_2MDP is to administer the drug prior to infection with virus. In the next experiments, the drug was delivered 1 day prior to virus inoculation. In agreement with a previous report, drug treatment 1 day prior to virus inoculation or on even the same day resulted in acute encephalitis and death in nearly all the drug recipients a few days p.i. (40). This outcome probably occurred because macrophages are key mediators of the initial, innate immune response to infection with MHV.

To prevent this outcome, small amounts of neutralizing anti-S antibody (12.5 μ l of MAb 5A13.5 and 5B19.2) were administered to dams previously immunized with live MHV-JHM. This amount, administered 3 days prior to virus inoculation, was by itself unable to protect suckling mice from acute encephalitis, but in conjunction with Ab generated in response to active immunization, it reduced the mortality in the $\text{Cl}_2\text{MDP-L}$ -treated group before day 15 p.i. from 68.4 to 14.5%. Even with this intervention, mortality in the drug-treated group was greater than in the untreated mice (Fig. 3), confirming the importance of macrophages in the initial response to MHV. Drug treatment did not appreciably affect virus growth in the infected CNS since infectious virus could be

FIG. 3. Effect of treatment with $\text{Cl}_2\text{MDP-L}$ on mortality. The drug was administered to mice 1 day prior to virus inoculation (14 treated and 18 control mice), and the mice were monitored daily for mortality. No difference in survival between control and drug-treated samples was observed when analyzed by Fisher's exact test (two tailed).

cultured from the brains and spinal cords of most control and $\text{Cl}_2\text{MDP-L}$ -treated mice (Table 1).

Liver and spleen samples were examined for the presence of macrophages by immunohistochemistry with anti-F4/80 Ab. As shown in Fig. 1B, depletion of macrophages from the liver was complete. The extent of depletion was quantitated by analysis of sections from the livers of nine pairs of control and $\text{Cl}_2\text{MDP-L}$ -treated mice. For this purpose, representative sections were stained for the presence of macrophages with F4/80 antibody and the number of positive cells per unit area was determined. Depletion was $98.8\% \pm 0.38\%$ complete. Similarly, splenic macrophages in the red pulp and marginal zone were also eliminated (data not shown), in agreement with previous results (2).

Examination of the spinal cords revealed strikingly different results (Fig. 1). Large numbers of macrophages/microglia were detected in control specimens, and treatment with $\text{Cl}_2\text{MDP-L}$ did not affect this result (Fig. 1D and E). The distributions of macrophages in the spinal cords of the control and drug-treated samples were very similar. To quantitate the number of macrophages, random fields from control and drug-treated

TABLE 2. Quantitation of demyelination in infected spinal cords

Treatment	% Demyelination (mean $\pm$ SE) ^a	Mean demyelination for treatment ^b
No drug	23.8 $\pm$ 3.5 17.5 $\pm$ 2.0 28.4 $\pm$ 3.0	23.2 $\pm$ 3.2
$\text{Cl}_2\text{MDP-L}$	22.0 $\pm$ 5.8 20.0 $\pm$ 4.3 36.7 $\pm$ 7.2	26.2 $\pm$ 5.3
$\text{Cl}_2\text{MDP-mnL}$	28.3 $\pm$ 1.1 33.9 $\pm$ 1.6 32.1 $\pm$ 1.7	31.5 $\pm$ 1.4

^a Demyelination was quantitated by a computer-based method as described in Materials and Methods. No statistical difference in demyelination between the control and either the $\text{Cl}_2\text{MDP-L}$ or the $\text{Cl}_2\text{MDP-mnL}$ -treated samples was detected when analyzed by Student's *t* test (two-tailed). Each value is the mean obtained from measurements on three to five sections of cord; three mice were used per treatment.

^b Mean values for each treatment.

samples from four control and six drug-treated mice were analyzed. Similar numbers of macrophages were detected in each set of samples (218 ± 30 macrophages/ mm^2 [mean $\pm$ standard error] in control mice and 186 ± 23 macrophages/ mm^2 in treated mice). Similarly, large areas of demyelination were present in the spinal cords of infected mice (Fig. 1G), in agreement with previous results (23). $\text{Cl}_2\text{MDP-L}$ treatment did not diminish the amount of demyelination (Fig. 1H). The amount of demyelination was quantitated by using computer-based image analysis technology (Table 2). For these measurements, three to five sagittal sections of spinal cord from each of three control mice and three $\text{Cl}_2\text{MDP-L}$ -treated mice were stained with LFB and processed as described in Materials and Methods. These quantitative analyses showed that depletion of blood-borne macrophages did not diminish the amount of demyelination present in the CNS of MHV-infected mice.

Depletion of macrophages with $\text{Cl}_2\text{MDP-mnL}$ 1 day prior to virus inoculation does not decrease the amount of demyelination. $\text{Cl}_2\text{MDP-mnL}$ but not $\text{Cl}_2\text{MDP-L}$ prevented the development of EAE in Lewis rats, although both drugs caused the depletion of macrophages from the liver and spleen (11). Next, mice were treated with $\text{Cl}_2\text{MDP-mnL}$ 1 day prior to infection with MHV and every 5 days thereafter. Results similar to those obtained with $\text{Cl}_2\text{MDP-L}$ treatment were obtained. In these experiments, mice were protected from acute encephalitis more completely than in the experiment in Fig. 3 and Table 1, with no mortality detected in the control group (Fig. 4A). Nevertheless, mortality was still greater in the drug-treated mice, and these mice exhibited significantly slower growth than did the control population (Fig. 4B). Depletion of macrophages from the liver was nearly complete ($98.9 \pm 0.75\%$ depletion) (Fig. 1C). However, large numbers of F4/80-positive macrophages/microglia were present in the spinal cords from $\text{Cl}_2\text{MDP-mnL}$ -treated mice (Fig. 1F) and extensive demyelination was detected in these samples (Fig. 1I). The number of macrophages was quantitated from the spinal cords of four control mice and five drug-treated animals, as described above. Similar numbers of macrophages were detected in the spinal cords of these animals (162 ± 19 macrophages/ mm^2 [mean $\pm$ standard error] in control mice and 202 ± 16 macrophages/ mm^2 in treated mice). The amount of demyelination was quantitated by computer-based image analysis technology as described above. The percentage of demyelination in the spinal

TABLE 1. Virus titers in control and $\text{Cl}_2\text{MDP-L}$ -treated mice^a

Treatment	No. of mice	No. (%) with virus detected in:		Virus titer ^b in:	
		Brain	Spinal cord	Brain	Spinal cord
None (control)	18	14 (78)	16 (89)	4.62 $\pm$ 0.23	4.51 $\pm$ 0.23
$\text{Cl}_2\text{MDP-L}$	14	9 (64)	13 (93)	4.21 $\pm$ 0.29	4.06 $\pm$ 0.26

^a The drug was administered to mice 1 day prior to virus inoculation. Surviving mice were harvested on day 21. Virus titers in these mice and in moribund mice euthanized between days 16 and 20 are included in the calculations.

^b Virus titers are expressed as geometric log₁₀ PFU/gram $\pm$ standard error. Only samples positive for virus are included in these calculations. There was no significant difference between control and drug-treated mice in the percentages of mice with virus detected in the brain and spinal cord ($P > 0.05$) when analyzed by Fisher's exact test (two tailed). The virus titers in animals with virus detected were not significantly different as determined by analysis of covariance.

FIG. 4. Effect of treatment with Cl₂MDP-mnL on mortality and morbidity. Drug was administered to mice 1 day prior to virus inoculation (11 treated mice and 6 control mice). The mice were monitored daily for mortality (A) and weighed every 5 days (B). No difference in survival between control and drug-treated samples ($P = 0.102$) was observed when analyzed by Fisher's exact test (two tailed). Weight gain differed significantly between the control and drug-treated mice on days 15 and 20 p.i. ($P < 0.0001$) as determined by a mixed-model analysis of variance.

cords of Cl₂MDP-mnL-treated mice was $31.5\% \pm 1.4\%$ (Table 2). These data suggest that there is an increase in demyelination in drug-treated mice, but this increase is not statistically significant because only a small number of mice were analyzed. Virus titers were equivalent in the brains and spinal cords of control and Cl₂MDP-mnL treated animals (Table 3).

Macrophages/microglia in the CNS of mice with acute encephalitis and chronic demyelination are infected with MHV-JHM. Treatment of TMEV-infected mice with Cl₂MDP-mnL prevented demyelination (26), but macrophages are the main reservoir of virus in mice chronically infected with TMEV. Therefore, depletion of macrophages also eliminates the most important site for virus replication. Based on the results described above, we predicted that macrophages would not be the predominant target for virus in mice persistently infected with MHV. Previously, we showed that 15 to 40% of the cells in the CNS of mice acutely or chronically infected with wild-type MHV-JHM were astrocytes, but we did not identify the phenotype of the remainder (20, 34). Macrophages are readily infected by MHV (12), and in the next experiments, we deter-

mined whether infection of macrophages/microglia accounted for a substantial fraction of the infected cells in the CNS of mice with either acute encephalitis or chronic demyelination. Wild-type MHV-JHM was used in these experiments. Macrophages/microglia were identified by immunohistochemistry with one of two specific Ab (F4/80 or Mac-1), and virus was identified by in situ hybridization with a ³⁵S-labeled antisense RNA probe as described in Materials and Methods. Infected macrophages/microglia could be detected in the CNS of mice with either acute encephalitis or the chronic demyelinating disease, but in neither case did these cells account for a large fraction of the infected cells. In mice with acute encephalitis, 11.5% of infected cells were macrophages/microglia, while in mice with the chronic demyelinating disease, 13.7% of infected cells were macrophages/microglia.

DISCUSSION

The objective of this study was to determine whether blood-borne macrophages are required for MHV-JHM-induced demyelination to occur. Our results showed that these cells are not absolutely required, since the presence of demyelination and appearance of macrophages/microglia in the infected CNS were not affected by treatment with either Cl₂MDP-L or Cl₂MDP-mnL. Treatment of MHV-infected mice with these drugs resulted in depletion of macrophages from the liver and spleen, in agreement with previous results (38). However, unlike animals with EAE or those infected with TMEV, this treatment did not abrogate the demyelinating process. Several possible explanations for this difference exist.

First, macrophages are not a major target for infection with MHV. In mice chronically infected with TMEV, macrophages are the main reservoir of virus in the white matter (15). Depletion of these cells greatly decreases the amount of infectious virus and viral antigen in the CNS (26). As a result, whether TMEV-induced demyelination is virus induced or immune system mediated, the consequence of macrophage depletion is a lower antigen burden and a decreased amount of demyelination. This scenario does not occur in MHV-infected mice.

TABLE 3. Virus titers in control and Cl₂MDP-mnL-treated mice^a

Treatment	No. of mice	No. (%) with virus detected in:		Virus titer ^b in:	
		Brain	Spinal cord	Brain	Spinal cord
None (control)	6	4 (67)	5 (83)	3.27 ± 0.42	4.42 ± 0.33
Cl ₂ MDP-mnL	8	6 (75)	8 (100)	3.35 ± 0.33	3.52 ± 0.23

^a The drug was administered to mice 1 day prior to virus inoculation. Surviving mice were harvested on day 21. Virus titers in these mice and in moribund mice euthanized between days 15 and 20 are included in the calculations.

^b Virus titers are expressed as geometric log₁₀ PFU/gram $\pm$ standard error. Only samples positive for virus are included in these calculations. There was no significant difference between control and drug-treated mice in the percentages of mice with virus detected in the brain and spinal cord ($P > 0.05$) when analyzed by Fisher's exact test (two tailed). The virus titers in animals with virus detected were not significantly different as determined by analysis of covariance, although the values for the spinal cord samples approached statistical significance ($P = 0.057$).

Second, $\text{Cl}_2\text{MDP-mnL}$ inhibits the adoptive transfer of EAE at an early stage in the disease process in Lewis rats and SJL/J mice (1, 10, 11, 35). In these animals, the inflammatory process begins in the presence of drug, since leukocytes pass from the blood across the endothelium into the perivascular and subarachnoid spaces. In SJL/J mice, macrophage depletion prevents T-cell migration across the blood-brain barrier and subsequent invasion of the parenchyma (11, 35). Antigen presentation by macrophages may be important for T-cell activation or survival in the CNS of these animals. Lymphocyte infiltration, in turn, may be important for glial cell activation and for initiation of the cascade of events that lead to demyelination. Alternatively, cytokines produced by macrophages may be important in upregulating immune function in glial cells. In Lewis rats, T-cell infiltration of the parenchyma is not affected by treatment with $\text{Cl}_2\text{MDP-mnL}$, but the subsequent steps in the demyelinating process are inhibited (10, 11).

In mice persistently infected with MHV, the load of viral antigen and infectious virus is high. Virus present only in the brain parenchyma is not believed to induce an immune response until antigen is processed by extraneural lymphatic tissue (4, 16, 30, 31), but the high load of viral antigen present makes it likely that this response is efficiently initiated. Additionally, dendritic cells, considered most important for antigen processing and subsequent activation of T cells, are not affected by treatment with $\text{Cl}_2\text{MDP-L}$ or $\text{Cl}_2\text{MDP-mnL}$ (3, 38). Thus, it is likely that T-cell activation is relatively normal in MHV-infected mice treated with either drug. The presence of activated T cells combined with large amounts of viral antigen in the CNS may facilitate the activation of perivascular macrophages and microglia.

In our experiments, mice were treated with drug every 5 days. This regimen should prevent repopulation of the CNS by hematogenously derived monocytes (2, 9, 36). Our results suggest that either perivascular macrophages or microglia, neither of which are depleted by treatment with $\text{Cl}_2\text{MDP-L}$ or $\text{Cl}_2\text{MDP-mnL}$, are able to substitute for monocyte-derived macrophages. In particular, they appear to serve as the final effector cells in the demyelinating process. Perivascular macrophages are bone marrow derived and function as antigen-presenting cells in pathological conditions and in the normal CNS (6). This population undergoes replacement with bone marrow-derived cells. Resident microglia, while also of hematopoietic origin, are a highly stable population of cells with a low turnover rate. They are difficult to distinguish from infiltrating or perivascular macrophages because they express similar phenotypic markers, with the exception of CD45. Microglia are reported to be CD45^{low} , whereas macrophages are $\text{CD45}^{\text{high}}$ (28). Recent results suggest that although microglia isolated from the normal CNS do not present antigen effectively, they become activated under pathological conditions, including infection with MHV-JHM, and are able to up-regulate MHC class I and class II expression (5, 27, 28). Under these conditions, they proliferate, become phagocytic, and are difficult to distinguish functionally from perivascular macrophages. As a result, we do not yet know which of these two cell types is most important in antigen presentation and myelin removal in MHV-infected mice in which blood-borne macrophages are depleted.

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