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 diphosphonate (Cl2MDP) or with N,N’-bis[3,5-bis[L-((aminiminomethyl)hydrazono)ethyl]phenyl decanedia-mide 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-
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 (21), unlike for TMEV-infected animals (15). Therefore, depletions 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-derivcd 17CL-1 cells as described previously (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^d 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 intra-nasally with either wild-type or CTL escape mutant virus (2.5 X 10^5 PFU) at 10 days of age and were nursed by dams previously inoculated with live MHV-JHM (21). Mice infected with wild-type virus developed hindlimb paralysis between 3 and 8 weeks p.i., whereas most mice infected with mutant 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 ven-tricle. Livers, spleens, brains, and spinal cords were harvested from these mice.

Tissue processing. For immunohistochemistry, samples were fixed with His-tochoice (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 mannansylated liposomes were constructed from phosphatidylcholine, cholesterol, and mannose as previously de-scribed (39). Briefly, 4.7 mg of phosphatidylcholine (Lipoid GmbH, Donald, 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 mannansylated liposomes, 0.7 mg of phosphatidylcholine and 10.8 mg of cholesterol were dissolved in chloroform and added to 3.6 mg of p-amino-phenol-a-o-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 CI-MDP (a kind gift of Boehringer GmbH, Mannheim, Germany) by gentle rotation for 10 min. Free CI-MDP was removed by rinsing the liposomes with sterile PBS and centrifuging them for 30 min at 25,000 X 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 CI-MDP-L and CI-MDP-mnL on MHV-induced demyelination was determined in two separate sets of experi-ments. In the first set of experiments, 14 suckling mice were treated with CI-MDP-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 CI-MDP-L 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-mac-rophage monoclonal antibody (Ab) against CD11b (Serotec, Oxford, England) recognizes a protein with homology to a family of hormone receptors (18). Rat anti-Mac-1 (clone M1/70) recognizes I-Ab and was obtained from M. Dailey, University of Iowa. Murine Ab recognizing major histocompatibility complex (MHC) class I antigen (anti-H-2K^d/H-2D^d [clone 20-8-4]) and MHC class II antigen (anti-I-A^d [clone 25-9-175]) 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. Bachmeier, The Scripps Research Insti-tute. Mannansylated goat anti-rat IgG 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 Super Frost/Plus Mi-croscope 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.) (1:10,000 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.

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-buffered-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 (1:200), anti-I-A^d Ab (1:500), or anti-I-A^d Ab (1:200) and anti-H-2K^d Ab (1:500) for 24 h at room 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 Lab-oratories) 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 hy-bridization with an antisense riboprobe for MHV RNA was combined with immunohistochemistry for F4/80 antibody (1:200) or Mac-1. Briefly, frozen sections from the spinal cords of mice were prepared, fixed in 2.5 to 4% phosphate-buffered-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, de-waxed, 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 the seven sections per slide were prepared for each spinal cord. 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 X200. Approximately 12 to 45 images were required to encompass an entire sagittal section. An image analysis program, VTrac (Image Analysis Facility, University of Iowa), was used to delineate myelinated and demyelinated areas. The marked areas were digitized, and two calculations were performed for each data set. 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...
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

Depletion of macrophages with Cl$_2$MDP-L at 7 days p.i. does not affect the extent of demyelination. As described above, treatment with liposomes containing Cl$_2$MDP, 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
may deplete intracellular iron stores or directly affect ATP metabolism. To determine if this reagent or its unmodified form (Cl$_2$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$_2$MDP 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 Cl$_2$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 Cl$_2$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$_2$MDP 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 μl 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 Cl$_2$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
cultured from the brains and spinal cords of most control and Cl2MDP-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 Cl2MDP-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% ± 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 Cl2MDP-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 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/mm² [mean ± standard error] in control mice and 186 ± 23 macrophages/mm² 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). Cl2MDP-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 Cl2MDP-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 Cl2MDP-mnL 1 day prior to virus inoculation does not decrease the amount of demyelination. Cl2MDP-mnL, but not Cl2MDP-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 Cl2MDP-mnL 1 day prior to infection with MHV and every 5 days thereafter. Results similar to those obtained with Cl2MDP-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 ± 0.75% depletion) (Fig. 1C). However, large numbers of F4/80-positive macrophages/microglia were present in the spinal cords from Cl2MDP-mnL-treated mice (Fig. 1F) and extensive demyelination was detected in these samples (Fig. 11). 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/mm² [mean ± standard error] in control mice and 202 ± 16 macrophages/mm² 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 Cl2MDP-L-treated mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>No. (%) with virus detected in:</th>
<th>Virus titerb in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Brain</td>
<td>Spinal cord</td>
</tr>
<tr>
<td>None (control)</td>
<td>18</td>
<td>14 (78)</td>
<td>16 (89)</td>
</tr>
<tr>
<td>Cl2MDP-L</td>
<td>14</td>
<td>9 (64)</td>
<td>13 (93)</td>
</tr>
</tbody>
</table>

*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.

*Virus titers are expressed as geometric log10 PFU/gram ± 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.

*Mean values for each treatment.

**TABLE 2. Quantitation of demyelination in infected spinal cords**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Demyelination (mean ± SE)a</th>
<th>Mean demyelination for treatmenta</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>23.8 ± 3.5</td>
<td>23.2 ± 3.2</td>
</tr>
<tr>
<td>Cl2MDP-L</td>
<td>22.0 ± 5.8</td>
<td>26.2 ± 5.3</td>
</tr>
<tr>
<td>Cl2MDP-mnL</td>
<td>28.3 ± 1.1</td>
<td>31.5 ± 1.4</td>
</tr>
</tbody>
</table>

*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 Cl2MDP-L or the Cl2MDP-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.

FIG. 3. Effect of treatment with Cl2MDP-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).
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.

Virus titers were equivalent in the brains and spinal cords of control and Cl$_2$MDP-mnL treated animals (Table 3).

**TABLE 3.** Virus titers in control and Cl$_2$MDP-mnL-treated mice*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>No. (%) with virus detected in:</th>
<th>Virus titer$^b$ in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Brain</td>
<td>Spinal cord</td>
</tr>
<tr>
<td>None (control)</td>
<td>6</td>
<td>4 (67)</td>
<td>5 (83)</td>
</tr>
<tr>
<td>Cl$_2$MDP-mnL</td>
<td>8</td>
<td>6 (75)</td>
<td>8 (100)</td>
</tr>
</tbody>
</table>

$^a$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 log$_{10}$ PFU/gram ± 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).

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 Cl$_2$MDP-L or Cl$_2$MDP-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.
Second, Cl2MDP-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 Cl2MDP-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 extraneuronal 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 Cl2MDP-L or Cl2MDP-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 Cl2MDP-L or Cl2MDP-mnL, are able to substitute for monocye-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 normal 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<sup>low</sup>, whereas macrophages are CD45<sup>high</sup> (28). Recent results suggest that although microglia isolated from the normal CNS do not present antigen efficiently, 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, 35).

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