The Role of Macrophages in the Pathogenesis of HSV-1 Induced Chorioretinitis in BALB/c Mice

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Purpose. To examine the effects of modification of immune effector cells, including macrophages, in the pathogenesis of herpes simplex virus retinitis in BALB/c mice.

Methods. Two intravitreal injections (2 μl each) of anti-CD11b monoclonal antibody (mAb) [13 μg/μl] were administered to the contralateral eyes of 10 BALB/c mice on days 6 and 8 after HSV inoculation into the right anterior chamber (AC) with HSV-1. A control group consisted of mice injected with anti-HLA-DR mAb in the same fashion. Specific macrophage depletion was performed in an additional group of 12 BALB/c mice by intravenous (IV) injection of dichloromethylene diphosphonate (Cl2MDP)-liposomes 7 days before AC HSV-1 inoculation into the eye. Control group consisted of mice receiving IV PBS-liposomes. Mice were clinically observed for 14 days postinfection, and the incidence of chorioretinal disease was confirmed by histopathologic studies.

Results. Intravitreal injections of anti-CD11b mAb produced a dramatic suppression of the contralateral retinal necrosis (2 of 10 mice) compared to 9 of 10 controls receiving an irrelevant antibody therapy (P < 0.05). Mice treated with IV Cl2MDP-liposomes also showed a significant inhibition of the development of contralateral chorioretinitis, with only 3 of 12 mice developing retinal disease compared to 9 of 12 mice from the control group (P < 0.05). FACS analysis performed on peripheral blood and spleen cells showed a significant depletion of Mac-1* cells of Cl2MDP-liposome-treated but not of PBS-liposome-treated mice (controls).

Conclusion. Intravitreal anti-CD11b mAb therapy, a broadly directed depletion strategy against many effector cells (macrophages, granulocytes, natural killer cells, and even cytotoxic T-cells) was most efficient in suppressing the HSV-1 induced contralateral disease. A more specific technique (IV Cl2MDP-liposome therapy) to deplete macrophages also produced a significant inhibition of HSV-1 induced contralateral chorioretinitis. These findings suggest that macrophages are important participants in the effector phase of the destructive inflammatory immune response induced by HSV-1 in the eye. Invest Ophthalmol Vis Sci. 1994;35:2990-2998.

The murine von Szily model of herpes simplex virus type 1 (HSV-1) chorioretinitis is produced by the inoculation of HSV-1 into one anterior chamber (AC) of susceptible BALB/c mice.1,2 Between 7 to 10 days after inoculation, the mice develop contralateral retinitis produced by inflammatory cells infiltrating the retina to clear herpes viral particles.3 The invading inflammatory cells consist mainly of macrophages, neutrophils, natural killer (NK) cells, and to a lesser extent T-cells.4 Although numerous studies provide evidence about the influence of T-cells on HSV-1 induced chorioretinitis in mice,5-7 it is not clear by which mechanisms cell-mediated immunity initiates the clinical and pathologic findings of the contralateral HSV-1 necrotizing chorioretinitis characteristic of the von Szily model. The functional role of the different immune effector cells, particularly macrophages, in this model has not been studied. We hypothesized that macrophages are involved in the development of the chorioretinal damage because they are present in the

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Macrophages in the Pathogenesis of HSV-1 Chorioretinitis

Inflamed retina and because they secrete numerous cytokines that play multiple roles in specific and non-specific inflammatory reactions by attraction of immunocompetent cells into the eye, producing inflammation and necrosis.8

The purpose of the present study was to analyze the effects of intravitreal anti-CD11b mAb on the different effector cells infiltrating the contralateral retina of BALB/c mice after AC inoculation with HSV-1 and to elucidate further macrophage participation in the pathogenesis of the disease by more specifically modifying macrophage populations.

MATERIALS AND METHODS

Animals

Six- to 8-week-old male BALB/cByJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were handled in accordance with the ARVO resolution on the use of animals in research.9

Virus

HSV-1, KOS strain was obtained from Dr. David Knipe (Harvard Medical School, Boston, MA) and was grown in Vero cells (American Type Tissue Collection, CCL 81, Rockville, MD) as previously described.10

Anti-CD11b Monoclonal Antibody Therapy

Because the inflammatory cells invading the contralateral retina after AC HSV-inoculation mainly consist of macrophages, neutrophils, NK cells, and cytotoxic T-cells, we studied the effects of anti-CD11b mAb on these cells in their participation in the contralateral retinal destruction. For this purpose, two doses (given on days 6 and 8 postinoculation [PI]) of 2 μl each of anti-CD11b mAb [15 μg/μl] (clone M1/70 Serotec, Oxford, UK), an antibody of the IgG2b isotype directed against macrophages, granulocytes, NK cells, and cytotoxic T-cells were injected (under general anesthesia) into the contralateral vitreous cavity of BALB/c mice via pars plana, using a 30-gauge needle. A group of control mice received the same dose of anti-HLA-DR mAb (Becton-Dickinson, Mountain View, CA) in a similar fashion.

Macrophage Depletion

Macrophage depletion was based on the intracellular activity produced by the liposome-encapsulated drug, dichloromethylene diphosphonate (Cl2MDP).11 This technique offers the study of functional aspects of macrophages in vivo by the administration of Cl2MDP-liposomes intravenously (IV) to selectively kill macrophages present in peripheral blood and the major organs of the reticuloendothelial system (spleen and liver) and subcutaneously (SC) for regional lymph node macrophage depletion.12,13 Large multilamellar liposomes containing dichloromethylene-diposphonate (Cl2MDP) (a kind gift of Boehringer-Mannheim, Mannheim, Germany) were prepared as previously described.14 Briefly, 75 mg of phosphatidylcholine (a kind gift of Lipoid KC, Ludwigshafen, Germany) and 11 mg of cholesterol (Sigma Chemical, St. Louis, MO) were dissolved in 20 ml methanol/chloroform (1:1). The organic phase was then removed by low vacuum rotary evaporation (37°C), and the lipid film was dispersed in 10 ml of an aqueous solution (phosphate-buffered saline [PBS]). To enclose the Cl2MDP, 2.5 g of the drug were dissolved in 10 ml of PBS in which the lipid film was dispersed and the preparations were kept for 2 hours at room temperature, sonicated for 3 minutes, and resuspended in 4 ml of PBS. For systemic macrophage depletion, BALB/c mice received one dose of 200 μl (1 mg) of Cl2MDP-liposomes intravenously. Submandibular lymph node depletion was performed by injecting a single dose of 50 μl (0.25 mg) of Cl2MDP-liposomes SC in the ipsilateral and contralateral submandibular regions. Control groups received either IV or SC PBS-liposome therapy, respectively.

Viral Inoculation

Mice were anesthetized with 2 mg of ketamine HCl (Ketalar, Parke-Davis, Morris Plains, NJ) and 0.4 mg of xylazine (Rompun, Mosby, Shaw, KS) intraperitoneally. The ipsilateral eye was then inoculated with 2.5 × 105 PFU of HSV-1 as described previously.15

Clinical Examination

Ipsilateral and contralateral eyes were examined with a binocular microscope on days 4, 6, 8, 10, 12, and 14 PI. The development of intraocular inflammation, particularly contralateral chorioretinitis, was evaluated in a masked fashion. Anterior chamber inflammatory reaction, pupillary and iris vessel dilation, cataract formation, and vitreal haziness were scored on a scale of 0 to 4+, as previously described.8

Histopathology

Ipsilateral and contralateral eyes were stained for light microscopy as previously described.5 Briefly, specimens were placed in Karnovsky’s fixative (1% paraformaldehyde, 1.25% glutaraldehyde, 0.13% sucrose, and 25 mM sodium phosphate in 150 mM cacodylate buffer, pH 7.2) and stored overnight at 4°C. They were then dehydrated in ascending concentrations of ethanol using the automated LKB Ultraprocessor (Broma, Sweden) and embedded in historesin (LKB-Produkter AB, Broma, Sweden). Two-micron sections were performed and stained with hematoxylin and eosin.

Immunohistopathology

Immunofluorescence staining of five representative contralateral eyes per group, blood smears, spleen,
Macrophages in the Pathogenesis of HSV-1 Chorioretinitis

RESULTS

Clinical Findings

To study the effects of depletion of macrophages and other immune effector cells that migrate to the contralateral eye after HSV-1 inoculation into the right AC, 10 mice received anti-CD 11b mAb intravitreally into the contralateral (left) eye. This treatment resulted in a dramatic suppression of contralateral retinal necrosis, 1 of 10 mice, compared to 9 of 10 control mice receiving an irrelevant antibody (anti-HLA-DR) therapy (Table 1).

The effects of specific systemic macrophage depletion were analyzed in mice treated with IV Cl₂MDP-liposomes (group 3). These mice showed a profound suppression of the development of contralateral chorioretinitis, with only 2 of 12 (16.6%) mice developing retinal disease by day 14 PI compared to 9 of 12 (75%) control mice receiving IV PBS-liposomes. Another group of mice (group 4) was depleted of submandibular lymph node macrophages by SC injections of Cl₂MDP-liposomes in the ipsilateral and contralateral submandibular regions. In this group, 8 of 12 (66.6%) mice developed contralateral retinal necrosis compared to 9 of 12 (75%) controls. The combination of IV and SC macrophage depletion (group 5) yielded similar results to those of group 3 (IV injection), with a statistically significant inhibition of the development of contralateral retinal necrosis (3 of 12) in mice treated with Cl₂MDP-liposomes compared to 10 of 12 (83.3%) IV and SC PBS-liposome-treated controls.

Histopathologic Observations

Light microscopy studies of contralateral eyes confirmed the clinical observations. The necrotizing chorioretinitis observed in affected eyes consisted of a marked mononuclear cell infiltration extending from the vitreous to all retinal layers, with total loss of the retinal architecture and focal areas of choroidal infil-
FIGURE 2. (Left) Preserved chorioretinal architecture with no inflammatory infiltrate seen in the contralateral eye of BALB/c mice treated with anti-CD11b mAb on days 6 and 8 after HSV-1 infection (hematoxylin and eosin, X64). (Right) Immunofluorescence staining of the same retina with anti-Mac-1 mAb showing absence of infiltrating Mac-1+ cells (X64).

...etration, usually under a retinal area with necrotic cystoid changes.

Only 2 of 10 (20%) mice treated with intravitreal anti-CD11b mAb developed contralateral retinitis compared to 9 of 10 (90%) anti-HLA-DR treated controls (P < 0.05).

In the group treated with IV Cl2MDP-liposomes only, 3 of 12 (25%) mice showed widespread retinal destruction compared to 9 of 12 (75%) control mice (P < 0.05). Histopathologic studies (hematoxylin and eosin) performed on the injected ipsilateral eyes of these mice showed no signs of retinal necrosis in any of the 12 eyes studied. Eight of 12 mice (66.6%) treated with SC Cl2MDP-liposomes in the submandibular region showed signs of contralateral retinal necrosis compared to 10 of 12 controls. The combination of IV and SC macrophage depletion resulted in only 3 of 12 (25%) mice with histopathologic patterns of chorioretinitis and retinal necrosis compared to 10 of 12 controls (P < 0.05).

**Immunofluorescence Studies**

Indirect immunofluorescence studies were performed on peripheral blood, spleen, and submandibular lymph nodes before inoculation with HSV-1 to compare the macrophage populations in Cl2MDP-liposome-treated mice with controls receiving PBS-liposomes (Fig. 1). Seven days after IV treatment with Cl2MDP-liposomes, peripheral blood and spleen showed markedly decreased numbers of Mac-1+ cells compared to controls (P < 0.05) (Fig. 1A). On the other hand, the submandibular lymph nodes from IV Cl2MDP-liposome-treated mice showed a similar number of macrophages compared to controls. In sharp contrast, SC injection of Cl2MDP-liposomes resulted in a significant decrease in the number of Mac-1+ cells...

FIGURE 3. (Left) Low-power micrograph showing extensive contralateral necrotizing chorioretinitis seen in BALB/c mice (control) treated with an irrelevant antibody (anti-HLA-DR mAb) on days 6 and 8 after HSV infection (hematoxylin and eosin, X25). (Right) Immunofluorescence staining of retina (square area delineated on Fig. 1A) with anti-Mac-1 mAb showing a positive Mac-1 cell infiltration and extensive retinal destruction (X160).
TABLE 2. FACS Analysis Showing the Percentage of Mac-1+ Cells in Blood, Spleen, and Submandibular Lymph Nodes 7 Days After Administration of Cl2MDP Liposomes

<table>
<thead>
<tr>
<th>Route of Liposome Administration</th>
<th>Peripheral Blood</th>
<th>Spleen</th>
<th>Submandibular Lymph Nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV Cl2MDP</td>
<td>0.8 ± 0.7*</td>
<td>1.2 ± 0.4*</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td>IV PBS</td>
<td>6.4 ± 0.2</td>
<td>7.9 ± 0.7</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>SC Cl2MDP</td>
<td>5.3 ± 0.9</td>
<td>9.2 ± 0.6</td>
<td>0.6 ± 0.2*</td>
</tr>
<tr>
<td>SC PBS</td>
<td>6.7 ± 0.6</td>
<td>10.1 ± 0.31</td>
<td>5.2 ± 0.6</td>
</tr>
<tr>
<td>IV + SC Cl2MDP</td>
<td>1.1 ± 0.4*</td>
<td>1.4 ± 0.3*</td>
<td>1.0 ± 0.4*</td>
</tr>
<tr>
<td>IV + SC PBS</td>
<td>5.8 ± 0.5</td>
<td>9.6 ± 0.8</td>
<td>6.5 ± 0.3</td>
</tr>
</tbody>
</table>

* Statistically significant: P < 0.05 (unpaired two-tailed Student's t-test). FACS analysis from five randomly chosen mice taken from each group.

FACS = Flow cytometry; IV = intravenous; SC = subcutaneous; Cl2MDP = dichloromethylene diphosphonate; PBS = phosphate-buffered saline.

in the ipsilateral and contralateral submandibular lymph nodes compared to controls receiving SC PBS-liposome therapy (Fig. 1B). Mice receiving a combination of IV and SC treatment with Cl2MDP-liposomes showed a significantly decreased number of Mac-1+ cells in the peripheral blood, spleen, and submandibular lymph nodes compared to controls (Fig. 1C).

Immunofluorescence studies performed on day 14 PI in contralateral eyes demonstrated that IV Cl2MDP-liposome and anti-CD11b mAb intravitreal therapies resulted in a preserved retinal architecture with few positive Mac-1+ cells compared to controls (Figs. 2, 3). Interestingly, positive anti-HSV-1 staining was found in both macrophage-depleted and a representative number (five eyes each) of control eyes, indicating the presence of viral particles in the chorioretinal layers even in unaffected eyes.

Flow Cytometry Analysis (FACS)

To determine the efficacy of IV Cl2MDP-liposome macrophage depletion, the percentage of Mac-1+ cells from peripheral blood, spleen, and submandibular lymph nodes present in mice after 7 days of IV, SC, or combination therapy with Cl2MDP-liposomes were analyzed by FACS (Table 2). Intravenous treatment with Cl2MDP-liposomes dramatically reduced the number of Mac-1+ cells in the peripheral blood (0.8 ± 0.7%) and spleen (1.2 ± 0.4%) compared to controls (P < 0.05). Subcutaneous submandibular injection of Cl2MDP-liposomes produced a significant decrease in the number of Mac-1+ cells in the submandibular lymph nodes (0.6 ± 0.2%), but the percentage of these cells remained unchanged in peripheral blood (5.3 ± 0.9%) and spleen (9.2 ± 0.6%).

Finally, the combination of IV and SC injections of Cl2MDP-liposomes resulted in a proportional reduction of Mac-1+ cells in blood (1.1 ± 0.4), spleen (1.4 ± 0.3%), and submandibular lymph nodes (1.0 ± 0.4%).

DISCUSSION

After AC HSV-1 inoculation, BALB/c mice are highly susceptible to the development of contralateral retinal necrosis. Although numerous studies provide evidence concerning the critical influence of T cells in the generation of and the protection from HSV-1 mediated contralateral chorioretinal necrosis, other effector immune cells, including macrophages, also appear to be essential in the effector phase of the disease.

We have previously shown that the tissue damage that leads to the clinical signs of HSV-1 chorioretinitis is caused by an inflammatory cell infiltrate predominantly composed of Mac-1+ cells (macrophages, neutrophils, NK cells, and to a lesser extent T-cells). Few reports are available on the role of these effector cells, particularly macrophages, in ocular inflammatory diseases.

We were initially interested in studying the effects of anti-CD11b mAb in stopping macrophages and other effector cells from locally participating in the inflammatory response seen in the contralateral retina. It has been described that HSV-1 appears in the contralateral eye between 6 and 8 days after anterior chamber injection, and by day 8 inflammatory cells (macrophages, granulocytes, NK cells, and cytotoxic T-cells) are detected in the eye. Therefore, anti-CD11b mAb, an antibody directed against these effector cells, was injected intravitreally before this first wave of inflammatory cells arrived to the eye. This resulted in a profound suppression of retinal necrosis with significant decrease in both the incidence and the severity of the retinitis, even though herpes simplex viral particles could be detected in the chorioretinal layers of unaffected eyes by indirect immunofluorescence.

The timing of the anti-CD11b mAb injection appeared to be critical for the inhibition of contralateral chorioretinitis. Suppression of retinal necrosis was ef-
effective only when the antibody was administered before the onset of clinical signs. Once the initial clinical signs of contralateral chorioretinitis were observed, anti-CD11b mAb did not alter the course of the disease (data not shown). This finding suggests that intravitreal anti-CD11b mAb therapy reduces the number of effector inflammatory cells and their availability for infiltration into the eye; simply preventing their direct participation in the inflammatory process seems to be sufficient to prevent the retinitis.

To investigate the role of macrophages in the pathogenesis of HSV-1 chorioretinitis, selective elimination of macrophages in vivo provides a useful tool. Several compounds, such as silica and carageenan, are known to affect macrophages, but these compounds also affect other immunocompetent cells. Because multilamellar liposomes are ingested exclusively by phagocytic cells (depending on their phospholipid concentration) and C12MDP, once it has been released in the circulation, has a short half-life and does not cross cell membranes, selective depletion of phagocytic cells using C12MDP-liposomes is not surprising. Indeed, this approach allows the selective removal of mononuclear phagocytes from heterogeneous spleen cell populations in vitro without effect on nonphagocytic spleen cells as measured by growth, protein production, antigen presentation, and antigen-specific T-cell proliferation. Dendritic cells appeared not to be removed from spleen tissue after IV treatment with C12MDP-liposomes. In addition, the number of dendritic cells isolated from C12MDP-liposomes treated animals was similar to that of dendritic cells obtained from controls. Moreover, dendritic cells from animals treated with empty (PBS) liposomes were equally effective in inducing in vitro primary CTL responses (Nair et al, submitted for publication). An important question concerns whether C12MDP-liposomes can produce depletion of neutrophil granulocytes because these cells are largely responsible for phagocytosis. There is no indication that neutrophil granulocytes are affected by C12MDP-liposomes. Neutrophils appeared neither morphologically, nor functionally to be affected by C12MDP-liposomes in vivo (Qian et al, submitted for publication).

In vitro experiments have confirmed this observation. After overnight in vitro culture of human neutrophils with C12MDP-liposomes (in a concentration comparable to that in the circulation of mice after IV administration of C12MDP-liposomes), no effects on the neutrophils could be demonstrated (unpublished results). Further studies have shown that the failure of C12MDP-liposomes to affect neutrophils could be explained by a low level of ingestion of liposomes. Both Dil-labeled C12MDP and Dil-labeled PBS-liposomes were minimally ingested by neutrophils (unpublished results). After IV administration, blood, liver, and spleen macrophages are selectively eliminated, whereas macrophages in other tissues remain unaffected. On the other hand, SC injection of C12MDP-liposomes produces depletion of macrophages in the regional lymph nodes where the injection is given.

In this study, clinicopathologic observations showed that depletion of peripheral blood and splenic macrophages alone or in combination with submandibular lymph node macrophages resulted in a significant decrease in the incidence and the severity of HSV-1-induced contralateral retinal necrosis. But depletion of submandibular lymph node macrophages alone did not influence the development of contralateral retinitis. These findings suggest that peripheral blood macrophages, and those residing in the spleen, importantly participate in the immune mechanisms that lead to retinal destruction in this model of infectious retinitis. In experiments performed by one of us (NVR) and colleagues, depletion of macrophages with C12MDP-liposomes produced marked attenuation of experimental allergic encephalomyelitis in Lewis rats. In this disease model, large numbers of macrophages have been found infiltrating the neural tissue in the central nervous system, and as in HSV-1-induced chorioretinitis, macrophages are thought to be major participants in the effector phase of the disease. Activated macrophages exert a number of inflammatory actions by the production of different cytokines such as TNF, IL-1, and γ-IFN. The release of arachidonic acid derivatives, complement components, reactive free radicals, cytolytic enzymes, and other proteases also contribute to local inflammatory reactions in the eye.

Even though the above findings suggest that macrophages are important participants of the inflammatory immune response to HSV-1 in the contralateral retina, it was not surprising to us that the intravitreal injection of anti-CD11b mAb was more effective in inhibiting the development of chorioretinal disease than was IV C12MDP-liposome therapy because CD11b is a cell surface marker shared by most effector cells of the immune system (macrophages, granulocytes, NK cells, and even cytotoxic T-cells). Therefore, anti-CD11b mAb has a broader spectrum in terms of cell recognition and, hence, a synergistic depletion effect. Furthermore, anti-CD11b mAb has been shown to inhibit a number of adhesion-dependent functions of both neutrophils and mononuclear cells, including chemotaxis, neutrophil aggregation, and binding of neutrophil and mononuclear cells to endothelium. In recent experiments, anti-CD11b/CD18 monoclonal antibody therapy has been shown to inhibit significantly the development of acute ocular inflammation in the endotoxin-induced uveitis model. Also, in vivo anti-CD11b antibody...
therapy has been shown to prevent the immunopathologic changes seen in viable moth-eaten bone marrow chimeric mice.\textsuperscript{39} In this mouse model, an autosomal recessive gene (me\textsuperscript{o}) occurred spontaneously as a point mutation of the hematopoietic cell protein tyrosine phosphatase in C57BL/6 mice. Homozygotes (me\textsuperscript{o}/ me\textsuperscript{o}) develop a chronic myelomonocytic inflammation, involving the accumulation of macrophages in lungs and skin resulting in interstitial pneumonitis and severe edema in the paws accompanied by thymic atrophy as well as NK cells and T-cell dysfunction. In vivo administration of anti-CD11b Ab significantly inhibited inflammation and restored NK and T-cell function by preventing extravasation and chemotaxis of macrophages into the affected tissues.

In conclusion, these results demonstrate that BALB/c mice can be effectively protected against development of HSV-1 contralateral retinal necrosis by giving intravitreal anti-CD11b mAb therapy before the onset of the clinical signs of the disease and also by IV injection of C\textsubscript{12}MDP before viral inoculation. Because macrophages are importantly affected and are a common denominator to both these unspecific (anti-CD11b mAb therapy) and specific (C\textsubscript{12}MDP-liposome therapy) depletion techniques, it seems reasonable to suggest that macrophages are important participants in the effector phase of the inflammatory immune response to HSV-1 induced contralateral chorioretinitis. Macrophages probably play a multifunctional role in the pathogenesis of the disease by direct cytotoxic action and indirectly by releasing chemotactic and immunoregulatory cytokines that finally contribute to retinal destruction.

**Key Words**

macrophages, C\textsubscript{12}MDP-liposomes, herpes simplex virus type 1, chorioretinitis, anti-CD11b monoclonal antibody

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