The Role of Macrophages in Acanthamoeba Keratitis

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Purpose. Macrophages are thought to be the first line of defense in many infectious diseases and are present in high numbers in corneas with Acanthamoeba keratitis. Conjunctival macrophage depletion was performed in an animal model of Acanthamoeba infection to determine the importance of macrophages in this disease.

Methods. Selective elimination of macrophages was achieved by repeated subconjunctival injection of liposomes containing dichloromethylene diphosphonate in a Chinese hamster model of Acanthamoeba keratitis.

Results. Macrophage depletion affected the incidence, severity, and chronicity of keratitis. The incidence of infection in normal animals was approximately 60% but rose to 100% on day 4 in animals treated with liposomes containing dichloromethylene diphosphonate (C12MDP–LIP). Moreover, the clinical appearance of the keratitis in the C12MDP–LIP group was much more severe than in animals treated with liposomes containing phosphate-buffered saline at all time points. There was also a major change in the chronicity of keratitis, with an earlier onset and a prolonged and chronic course in the C12MDP–LIP treated hamsters.

Conclusions. The profound exacerbation of Acanthamoeba keratitis in hamsters treated with C12MDP–LIP strongly suggests that macrophages play an important role in corneal infection with Acanthamoeba trophozoites, probably by acting as a first line of defense and eliminating significant numbers of Acanthamoeba trophozoites. Invest Ophthalmol Vis Sci. 1996;37:1271–1281.

Acanthamoeba keratitis is a potentially sight-threatening ocular infection caused by pathogenic free-living amoebae. This disease is characterized clinically by ulceration of the corneal epithelium, edema, and necrosis of the stroma. Diagnosis and treatment are often problematic, and infection can produce scarring of such severity that keratoplasty is required to restore normal vision. Acanthamoeba are distributed widely in the environment and can be isolated from swimming pools, fresh water, soil, dust, air-conditioning ducts, and the nasopharyngeal mucosa of healthy persons. Therefore, one might suspect that exposure is common. Contact lens wear generally is accepted to be the predominant risk factor and is practiced by more than 24,000,000 people in the United States alone, yet the occurrence of the disease is very low. Therefore, it seems likely that additional risk factors are important in the development of this disease.

Previous experiments have demonstrated that besides Acanthamoeba-contaminated contact lenses, abrasion (i.e., trauma) of the corneal epithelium is necessary to induce keratitis in animal models. Another potentially important factor in this disease is the role of the host’s immune response. Although little is known about the immune effector mechanisms against Acanthamoeba during ocular infection, histopathologic studies on the course of Acanthamoeba keratitis in experimental animals have shown that macrophages are present during the initial stages of infection. By contrast, macrophages have been observed in Acanthamoeba keratitis lesions in some patients.
but are conspicuously absent in others.10 It has been demonstrated that macrophages display chemotactic responses to Acanthamoeba antigens and can readily kill Acanthamoeba trophozoites in vitro.11 Because macrophages are thought to serve as a first line of defense against a wide variety of protozoal and bacterial infections, it is reasonable to predict that they also limit the clinical course of Acanthamoeba keratitis. Thus, the purpose of the current study was to determine the role of macrophages in Acanthamoeba keratitis and to determine whether paralyzing the conjunctival macrophage system would change the course of the disease to a more chronic form.

One approach to study the functional aspects of macrophages in vivo is to investigate the influence of macrophage depletion. Recently, a method was developed for selective elimination of macrophages by intravenous injection of liposomes containing dichloromethylene diphosphonate (C12MDP–LIP),12 a drug used to treat osteolytic bone diseases.13 Encapsulation of C12MDP by liposomes and phagocytosis of these liposomes is crucial for this technique. Because free C12MDP is not toxic by itself,14 C12MDP–LIP will only kill phagocytizing cells. After phagocytosis, the phospholipid bilayers of the liposomes are disrupted by lysosomal enzymes of the macrophage, and the drug is released into the cell, causing death of the macrophage either by depletion of intracellular iron or by affecting the ATP metabolism directly.14 Nonphagocytic cells are not affected by C12MDP–LIP.15 Neither dendritic cells, such as Langerhans cells, nor polymorphonuclear (PMN) leukocytes appear to be removed or functionally affected by C12MDP–LIP.16 This technique has proven to be effective in depleting macrophages from specific organs and tissues; recently, a functional effect was elegantly shown by Van der Veen et al,18 who could prevent corneal allograft rejection or functionally affected by C12MDP–LIP.16 This technique involves animals with preexisting lesions or anomalies. All procedures were performed on the right eye. The left eyes were not manipulated. All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**METHODS**

**Animals**

Chinese hamsters were purchased from Cytogen Research and Development (West Roxbury, MA) and Harlan CPB (Zeist, The Netherlands). Animals were used when they were 4 to 6 weeks of age, and all corneas were examined before the experiments to exclude animals with preexisting lesions or anomalies. All procedures were performed on the right eye. The left eyes were not manipulated. All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Parasite Cultivation**

Acanthamoeba castellanii, originally isolated from a diseased human cornea, was obtained from American Type Culture Collection (ATCC #30868; Rockville, MD) and grown axenically in peptone–yeast–glucose (PYG) medium as previously described.19

**Contact Lens Preparation**

Contact lenses were prepared from Spectra/Por dialysis membrane tubing (Spectrum Medical Industries, Los Angeles, CA) using a 3-mm trephine. Sterile dialysis tubing lenses (DTL) were incubated at 35°C for 24 hours with A. castellanii in 200 μl of PYG medium at a concentration of 3 × 10⁶ organisms/ml (50% trophozoites, 50% cysts) in sterile 96-well microtiter plates (Costar, Cambridge, MA). Attachment of parasites to the DTL was verified microscopically using a "hanging drop" technique.20

**Liposome-Encapsulated Dichloromethylene Diphosphonate**

Multilamellar liposomes were prepared as described earlier.21 Briefly, 8 mg cholesterol and 86 mg phosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany) were dissolved in 10 ml of chloroform in a round-bottomed flask. After low-vacuum rotary evaporation at 37°C, a thin film was formed on the inner surface of the flask. This film was then dispersed by gentle rotation for 10 minutes in phosphate-buffered saline (PBS) for the preparation of PBS-containing liposomes (PBS–LIP). Other liposome preparations were labeled with the lipophilic fluorescent dye, Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (D-282; Molecular Probes, Eugene, OR), as previously described.22 For clodronate liposomes (C12MDP–LIP), 2.5 g C12MDP (Clodronate; a gift from Boehringer Mannheim GmbH, Mannheim, Germany) was dissolved in 10 ml PBS. The suspension was kept for 2 hours at room temperature and sonicated for 3 minutes at 20°C. To remove free C12MDP, the liposomes were washed twice by centrifugation in PBS at 100,000g for 30 minutes and resuspended in 4 ml of PBS that contained approximately 20 mg of C12MDP. Each 100 μl of C12MDP–LIP suspension contained 1 mg of clodronate. C12MDP–LIP and PBS–LIP were stored at 5°C and used within 7 days of preparation. All liposomes, regardless of their contents, ranged in size from 100 nm to 3 μm in diameter.

**In Vivo Corneal Infection**

Acanthamoeba keratitis was induced in the Chinese hamster as described previously.4 Chinese hamsters were anesthetized with ketamine (100 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) injected intra-
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peritoneally. Topical anesthesia consisted of proparacaine (Alcon Laboratories, Fort Worth, TX). Twenty-five percent of the corneal surface was abraded gently under an operating microscope with a sterile cotton applicator before application of the parasite-laden DTL. After application of the DTL, the eyelids were closed by tarsorrhaphy with 10-0 Mersilene sutures (Ethicon, Somerville, NJ). The eye was exposed to the parasite-laden lens for 7 days. No topical or systemic antibiotics were used.

**In Vitro Effect of C12MDP-LIP on Macrophages**

The capacity of peritoneal macrophages to ingest liposomes and to be killed by C12MDP-LIP was evaluated in vitro. Peritoneal macrophages were harvested from five Chinese hamsters by the collection of peritoneal exudate. Each hamster received 3 ml of 2.5% thioglycollate injected intraperitoneally 4 days before sacrifice. Immediately after sacrifice, the peritoneal cavities were rinsed five times with 10 ml of Hank’s balanced salt solution (HBSS) under aseptic conditions. The cells were then washed three times in HBSS and suspended in complete RPMI 1640 medium. Macrophage suspensions either were used immediately in chromium-release assays (see next paragraph) or were added to individual wells in 96-well microtiter culture plates (1 × 10⁷ macrophages/well) and incubated with 50 μl of fluorescent liposomes for 4 hours at 37°C. Culture wells were washed three times with cold PBS, plates were chilled on ice for 5 to 10 minutes, and macrophages were removed by vigorous pipetting with PBS. Macrophages were then examined by fluorescent microscopy. The percent of macrophages containing one or more fluorescent liposomes was calculated.

The efficacy of C12MDP-LIP in killing macrophages was determined by a chromium-release assay. Peritoneal macrophage suspensions (1 × 10⁷ cells/ml) were incubated in 200 μCi ⁵¹Cr at 37°C for 1.5 hours. The cells were again washed three times in HBSS and resuspended in RPMI 1640 medium at 1 × 10⁶/ml. Four groups of five wells containing 1 × 10⁵ cells in 100 μl were prepared in a 96-well microtiter plate for incubation. The first group received 100 μl of PBS (spontaneous release control), the second received 50 μl of Hematall (Fisher Scientific, Pittsburgh, PA) and 50 μl PBS (maximal release control), the third received 100 μl of C12MDP-LIP, and the fourth received 100 μl of PBS-LIP. After 24 hours of incubation at 37°C, the plate was centrifuged for 5 minutes at 1000 rpm, and 100 μl from each well was counted in a gamma counter (Gamma-Safe Tm Analytic, Elk Grove Village, IL). Percent killing for the PBS and C12MDP liposome wells was calculated by the formula:

\[
\% \text{ killing} = \frac{\text{Experimental CPM} - \text{Spontaneous CPM}}{\text{Maximal Release CPM} - \text{Spontaneous CPM}} \times 100
\]

Results were reported as percent viable cells at 24 hours (100 − percent killing).

**Effect of C12MDP-LIP on Parasite Viability**

The effect of C12MDP-LIP on the viability of *Acanthamoeba* trophozoites was determined in vitro. Three sets of five 100-μl aliquots of *Acanthamoeba castellanii* 30868 trophozoites were prepared at a concentration of 5 × 10⁶ parasites/ml in PYG medium and placed in 96-well microtiter plates. The first group received 100 μl of PBS, the second group received 100 μl of C12MDP-LIP, and the third group received 100 μl of PBS-LIP. After 24 hours of incubation at 37°C, each well was agitated by repeated pipetting, and 100 μl was removed for counting. Each sample was diluted 1:10 in trypan blue (0.5%), and viable parasites (based on trypan blue exclusion) were counted in a hemocytometer. Percent viable parasites for each group was calculated by the following formula:

\[
\% \text{ viable parasites} = \frac{\text{Experimental Group}}{\text{PBS-LIP Group}} \times 100
\]

Results were reported as percent viable parasites at 24 hours.

**Macrophage Depletion**

Depletion of conjunctival–limbal macrophages was established by subconjunctival injection of C12MDP-LIP. Briefly, animals were anesthetized with ketamine 100 mg/kg intraperitoneally, and proparacaine (Alcon Laboratories) was used as a topical anesthetic. Under an operating microscope, the conjunctiva was lifted and 0.05 to 0.1 ml of the C12MDP-LIP suspension was injected into the bulbar conjunctiva using a 30-gauge needle mounted on a 1-ml tuberculin syringe. Injection of the C12MDP-LIP suspension resulted in a bleb around the injection site. To obtain a more equal distribution of the suspension around the limbus, we divided the doses by injecting at three different sites around the limbus until we obtained a circular conjunctival bleb. The total inoculum contained 0.5 to 1 mg of C12MDP. This procedure was performed four times at 2-day intervals. Animals were infected with parasite-laden contact lenses 48 hours after the last injection, as described above. Control hamsters were injected with PBS-LIP liposomes before infection. Additional control groups consisted of hamsters infected without any additional treatment as a baseline control and a group of animals treated with
C12MDP–LIP and fitted with sterile lenses. This group was included to evaluate the possibility that the bacterial flora, normally present in the eye, could induce keratitis because of reduced resistance caused by the C12MDP–LIP treatment.

**Clinical Observations**

Sutures and DTL were removed 7 days after tarsorrhaphy. Experimental and contralateral eyes were observed under a dissecting microscope for clinical signs of keratitis. Animals were observed three to four times per week throughout the course of the study, and the severity of keratitis was scored according to the degree of epithelial ulceration, stromal infiltration, corneal edema, and corneal neovascularization. Numerical values were assigned based on previously defined criteria for corneal ulceration, stromal infiltration and edema, and neovascularization.1

**Isolation of Parasites**

To confirm that the keratitis in the various experimental groups was caused by *Acanthamoeba* infection and was not the result of bacterial infection, contact lenses were removed from the eyes on days 4 and 7 and cultured for the isolation of *A. castellanii* trophozoites on a lawn of *Escherichia coli* (ATCC #25922) cultured on nonnutrient agar. *E. coli* cultures were observed for the development of characteristic amoeba trails.20,23 Corneal scrapings were prepared from eyes demonstrating clinical signs of infection at the time of removal of the DTL and examined for the presence of bacteria and *Acanthamoeba*. At different time points after infection, animals were killed, eyes were enucleated, corneas were homogenized in saline using a glass tissue grinder, and the homogenate was cultured for the isolation of bacteria and *A. castellanii*. *Acanthamoeba* were isolated by culturing the homogenate on lawns of *E. coli* as described above. Bacteria were isolated by culturing corneal specimens on BBL *Brucella* agar (Becton Dickinson Microbiological Systems, Cockeysville, MD). Bacterial identification was made using bacterial identification test panels (Biolog Micro Station System; Biolog, Hayward, CA). Animals whose cultures were positive for pathogenic bacteria were excluded.

**Histology**

Eyes were collected for histology on days 4, 7, and 14 after infection. Corneal specimens were fixed in Carson’s formalin, progressively dehydrated to 100% ethanol, and embedded in paraffin. Four-micrometer sections were cut and stained with hematoxylin and cosin.

**Enzymohistology: Acid Phosphatase Staining**

To verify depletion of or changes in the periocular macrophage population, eyes were processed and stained for endogenous acid phosphatase activity. Chinese hamsters treated with four subconjunctival injections with C12MDP–LIP, PBS–LIP, and PBS as described above were killed by cervical dislocation under anesthesia 2 days after the last injection, and the eyes were enucleated.

The enucleated eyes were washed in PBS, embedded in Tissue-tek (OCT compound; Miles Scientific, Naperville, IL) in a small plastic cup, frozen in liquid nitrogen after immersion in isopentane cooled with liquid nitrogen, and stored at —80°C. Meridional cryostat sections (6 μm) were made in the middle of the eye at —20°C and mounted on chromealum–gelatin-coated glass slides. Sections were allowed to air dry for at least 30 minutes, fixed in acetone for another 10 minutes, and air dried for 10 minutes. To demonstrate endogenous acid phosphatase activity of macrophages, slides were incubated with naphthol AS-BI phosphate and pararosaniline for 35 minutes at 37°C.24 Spleen tissue was used as a positive control. All slides were counterstained lightly with hematoxylin before mounting with Kaisers’ gelatin (Merck, Darmstadt, Germany).

Using a normal light microscope, the effect of C12MDP–LIP treatment on acid phosphatase-positive resident macrophages was determined for several parts of the eye, including corneal epithelium, corneal stroma (including the limbus), ciliary body, iris, bulbar conjunctiva, and subconjunctival tissue.

**RESULTS**

**Effect of C12MDP–LIP and PBS–LIP on Macrophages and Acanthamoeba Trophozoites**

Before initiating in vivo experiments, it was important to determine whether macrophages actively ingested liposomes and to what degree C12MDP–LIP would eliminate macrophages. Within 2 hours of in vitro exposure to fluorescent liposomes, >95% of the macrophages contained ingested fluorescent liposomes (data not shown). Macrophages also rapidly ingested nonfluorescent C12MDP–LIP and PBS–LIP (data not shown). Peritoneal macrophages and *Acanthamoeba* trophozoites also were exposed to either C12MDP–LIP or PBS–LIP, and their viability was determined 24 hours later. Although PBS–LIP did not affect macrophage viability, C12MDP–LIP produced extensive killing of macrophages (Table 1). Only 16% of the macrophages incubated with PBS–LIP perished, whereas 99% of the C12MDP–LIP-treated macrophages were killed. By contrast, both PBS–LIP and
The role of macrophages in the development of corneal ulceration and, in some cases, perforation of the cornea. C12MDP-LIP treatment produced almost total depletion of macrophages in the cornea—limbus and conjunctiva that persisted for at least 1 week after the final subconjunctival inoculation of C12MDP-LIP. By contrast, C12MDP-LIP treatment had no discernible effect on the number or staining intensity of acid phosphatase-positive cells in the corneal epithelium, corneal stroma, ciliary body, or iris.

Depletion of macrophages by C12MDP-LIP had a profound effect on the incidence, severity, and chronicity of keratitis. The incidence of infection in normal animals was approximately 60% but rose to 100% in C12MDP-LIP-treated animals (Table 2). Moreover, the clinical appearance of the keratitis in the C12MDP-LIP group was much more severe than in the PBS-LIP group at all time points, resulting in corneal ulceration and, in some cases, perforation of the cornea.

In addition to exacerbating the incidence and severity of keratitis, C12MDP-LIP treatment also altered the chronicity of the keratitis in the C12MDP-LIP-treated animals. Corneal disease was prolonged in the C12MDP-LIP-treated hamsters: Severe keratitis was present in 73% of the animals at day 14 and remained moderate in 66% of the animals at day 39. By contrast, only 15% of the PBS-LIP hamsters had mild keratitis after 14 days, and all were clinically normal by day 28.

**Histology**

Whole eyes were collected from PBS-LIP and C12MDP-LIP groups on days 4, 7, and 14 for histopathologic analysis. The histopathologic features of the eyes from C12MDP-LIP-treated Chinese hamsters were markedly and consistently more severe than those of untreated controls and PBS-LIP-treated animals. Histopathologic changes were found only in those eyes with clinically present gross lesions.

At day 4, there were no clinically significant changes or histopathologic sequelae in the eyes of hamsters treated with PBS-LIP before corneal infection (Fig. 3). By contrast, the C12MDP-LIP group was characterized by focal epithelial ulceration and PMN exocytosis (Fig. 4). The corneal stroma of this group contained marked lamellar connective tissue disruption and edema, severe PMN cell infiltration, and diffuse stromal thickening (Table 3). No macrophages were observed in the stroma or epithelium.

By day 7, histopathologic changes in the two groups were similar qualitatively and varied only in severity of inflammation. Epithelial changes consisted of ulceration and necrosis, PMN exocytosis, and focal thickening and spongiosis. Stromal changes included lamellar connective tissue disruption, PMN infiltration, neovascularization, thickening, and edema (Fig. 5). Only occasional macrophages were observed. Interestingly, lymphocytes occasionally were seen in the anterior chamber and adhering to the corneal endothelium in both C12MDP-LIP- and PBS-LIP-treated animals (Figs. 5, 6). The overall histopathologic changes were mild to moderate for the PBS-LIP group, and the clinical and histologic character of these lesions was consistent with that of untreated Chinese hamsters at this time point (Fig. 6). However, the changes in the C12MDP-LIP-treated group were substantially more severe than either PBS-LIP-treated or untreated Chinese hamsters (Table 3).

PBS-LIP-treated eyes exhibited only mild lesions by day 14, and many eyes that had previously exhibited lesions had returned to normal (Fig. 7). The corneal epithelium of affected eyes was mildly and diffusely thickened, and the stroma contained occasional PMNs and small lymphocytes and exhibited mild edema. By contrast, C12MDP-LIP-treated eyes still exhibited moderate to severe clinical and histologic changes (Fig. 8). The corneal epithelium of this group was acanthotic, corrugated, and spongiotic, and it contained micropustules and inflammatory crusts.

### TABLE 1. Effect of Liposomes on Macrophages and *Acanthamoeba* Trophozoites

<table>
<thead>
<tr>
<th>Cell</th>
<th>Untreated</th>
<th>PBS-LIP</th>
<th>C12MDP-LIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>100 ± 0</td>
<td>69 ± 12</td>
<td>16 ± 6</td>
</tr>
<tr>
<td><em>Acanthamoeba</em></td>
<td>100 ± 0</td>
<td>62 ± 11</td>
<td>64 ± 14</td>
</tr>
</tbody>
</table>

* Viability of peritoneal macrophages and trophozoites was determined by 31Cr release and trypan blue exclusion, respectively. Macrophages and trophozoites were incubated in PYG alone (untreated) or with PBS-LIP or C12MDP-LIP for 24 hours at 37°C. Results are expressed as mean ± SEM. Similar results were obtained in two independent experiments.

PBS = phosphate-buffered saline; LIP = liposome; PYG = peptone-yeast-glucose.
FIGURE 1. Photomicrograph of a bulbar conjunctiva from a hamster treated with PBS-LIP. Red staining is indicative of acid phosphatase positive cells (macrophages). Similar results were found with untreated corneas and corneas injected with PBS. There were five animals in each group. Limbus is not present in this photograph. Bar = 20 μm. PBS = phosphate-buffered saline; LIP = liposome.

FIGURE 2. Photomicrograph of a cornea from a hamster treated with subconjunctival injections of C12MDP-LIP. Eyes were removed and stained 48 hours after fourth subconjunctival injection. Note the absence of positive staining for acid phosphatase. Similar results were found in all five animals in this group. The limbus is not present in this photograph. Bar = 20 μm. LIP = liposome.
TABLE 2. Summary of Clinical Data of Acanthamoeba Keratitis in C12MDP-LIP and PBS-LIP Pretreated Eyes

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>% Infection*</th>
<th>Mean Infection Score†</th>
<th>Infection Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12MDP-LIP</td>
<td>100 (12/12)</td>
<td>1.20</td>
<td>Moderate</td>
</tr>
<tr>
<td>PBS-LIP</td>
<td>62 (8/13)</td>
<td>0.35</td>
<td>Mild</td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12MDP-LIP</td>
<td>88 (30/34)</td>
<td>2.44</td>
<td>Severe</td>
</tr>
<tr>
<td>PBS-LIP</td>
<td>59 (20/34)</td>
<td>0.82</td>
<td>Moderate</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12MDP-LIP</td>
<td>73 (8/11)</td>
<td>2.16</td>
<td>Severe</td>
</tr>
<tr>
<td>PBS-LIP</td>
<td>15 (2/13)</td>
<td>0.29</td>
<td>Mild</td>
</tr>
<tr>
<td>Day 28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12MDP-LIP</td>
<td>66 (4/6)</td>
<td>1.56</td>
<td>Moderate</td>
</tr>
<tr>
<td>PBS-LIP</td>
<td>0 (0/10)</td>
<td>0.00</td>
<td>None</td>
</tr>
<tr>
<td>Day 39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12MDP-LIP</td>
<td>66 (4/6)</td>
<td>1.19</td>
<td>Moderate</td>
</tr>
<tr>
<td>PBS-LIP</td>
<td>0 (0/10)</td>
<td>0.00</td>
<td>None</td>
</tr>
</tbody>
</table>

* Values in parentheses represent the number of hamsters in each group.
† Mean of all infected animals in that group, mean clinical score for each animal scored for epithelial defects, stromal infiltration, stromal edema, and neovascularization (scale 1–4).

PBS = phosphate-buffered saline; LIP = liposome.

inflammatory population in the stroma changed to include plasma cells and small lymphocytes in addition to the PMNs. Additionally, the stromal connective tissue was still disrupted and neovascularized.

DISCUSSION

The purpose of this study was to determine the role of macrophages in Acanthamoeba keratitis by selective elimination of the conjunctival–corneal macrophages through C12MDP–LIP treatment. Repeated subconjunctival injections with C12MDP–LIP had a profound effect on Acanthamoeba keratitis. Not only did the incidence of infection rise sharply, the course of the keratitis was changed dramatically as well, with an earlier onset of the clinical signs, a more severe keratitis, and a prolonged, more chronic course without complete resolution.

The profound exacerbation of Acanthamoeba keratitis in hamsters treated with C12MDP–LIP strongly suggests that macrophages play an important role in corneal infection with Acanthamoeba trophozoites. It is possible that C12MDP–LIP treatment prevented the generation of an Acanthamoebiaspecific immune response by pre-

FIGURE 3. Photomicrograph of a cornea from a Chinese hamster injected subconjunctivally with PBS–LIP before challenge with parasite-laden contact lenses. Eye was removed 4 days after parasite challenge. Note the extensive stromal swelling. Bar = 20 μm. PBS = phosphate-buffered saline; LPS = liposome.

FIGURE 4. Photomicrograph of a cornea from a Chinese hamster treated by subconjunctival injections of C12MDP–LIP. Eye was removed 4 days after parasite challenge. Note the extensive stromal swelling. Bar = 20 μm. LIP = liposome.
TABLE 3. Histologic Analysis of Corneas with *Acanthamoeba* Keratitis After Treatment With C12MDP-LIP or PBS-LIP

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Epithelium</th>
<th>Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td></td>
</tr>
<tr>
<td>C12MDP-LIP</td>
<td>Focal ulceration</td>
<td>Lamellar connective tissue disruption</td>
</tr>
<tr>
<td></td>
<td>Epithelial necrosis</td>
<td>Severe PMN infiltrate, no macrophages</td>
</tr>
<tr>
<td></td>
<td>Focal PMN crust formation</td>
<td>Diffuse thickening and edema</td>
</tr>
<tr>
<td>PBS-LIP</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td></td>
</tr>
<tr>
<td>C12MDP-LIP</td>
<td>Focal ulceration and necrosis</td>
<td>Lamellar connective tissue disruption</td>
</tr>
<tr>
<td></td>
<td>Focal PMN crust formation</td>
<td>Severe PMN infiltrate, no macrophages</td>
</tr>
<tr>
<td></td>
<td>Focal thickening and spongiosis</td>
<td>Severe edema</td>
</tr>
<tr>
<td>PBS-LIP</td>
<td>Focal epithelial necrosis</td>
<td>Lamellar connective tissue disruption</td>
</tr>
<tr>
<td></td>
<td>Occasional PMN exocytosis</td>
<td>Mild to moderate PMN, occasional macrophages</td>
</tr>
<tr>
<td></td>
<td>Focal thickening and spongiosis</td>
<td>Diffuse edema</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td></td>
</tr>
<tr>
<td>C12MDP-LIP</td>
<td>Multifocal micropustule formation</td>
<td>Lamellar connective tissue disruption</td>
</tr>
<tr>
<td></td>
<td>Spongiosis and crusting</td>
<td>Severe PMN and plasma cell infiltrate</td>
</tr>
<tr>
<td></td>
<td>Diffuse acanthisis and corrugation</td>
<td>No macrophages</td>
</tr>
<tr>
<td>PBS-LIP</td>
<td>Diffuse, mild thickening</td>
<td>Mild to moderate neovascularization</td>
</tr>
</tbody>
</table>

LIP = liposome; PBS = phosphate-buffered saline; PMN = polymorphonuclear neutrophil.

venting antigen presentation to T cells by conjunctival macrophages. A disruption of a potentially protective immune response could account for the more severe and chronic corneal infections that occurred in the C12MDP−LIP-treated hamsters. However, recent results in porcine55 and hamster (manuscript in preparation) models indicate that corneal infections with *Acanthamoeba* do not elicit protective immunity; as a result, hosts are susceptible to reinfection even after resolution of an initial episode of *Acanthamoeba* keratitis. Moreover, the recrudescence that occurs in patients with *Acanthamoeba* keratitis supports the notion that protective immunity is not evoked by corneal infection with *Acanthamoeba* trophozoites in humans. A marked difference in the severity of keratitis in C12MDP−LIP- and PBS−LIP-treated hamsters is evident as early as day 4 of infection, a time seemingly too early for the generation and expression of conventional T cell-dependent effector mechanisms. The most likely explanation to account for the exacerbation of *Acanthamoeba* keratitis in hosts treated with C12MDP−LIP is that macrophages act as a first line of defense and eliminate significant numbers of *Acanthamoeba* trophozoites. This would prevent the disease from becoming chronic because the extensive attack of the macrophages early in the infection could prohibit the spread and invasion of the *Acanthamoeba*. In turn, this would limit the opportunity for the parasite to infiltrate the cornea and eventually prevent encystment of the parasite in the corneal stroma. This hypothesis of the first line of defense by macrophages is supported by previous studies11 showing that macrophages demonstrate a strong chemotactic response to *Acanthamoeba* and can directly kill trophozoites in vitro. Although the absence or paucity of macrophages in corneal biopsy specimens and corneal buttons from patients with *Acanthamoeba* keratitis who underwent penetrating keratoplasty calls into question the role of macrophages in controlling *Acanthamoeba* infections of the cornea,10 it should be noted that most histopathologic studies on patients with *Acanthamoeba* keratitis who have examined corneal specimens during the latter stages of disease and not during the acute phase.6-10 We suspect that if macrophages serve as an important barrier to corneal infection, they would exert their effect by preventing the initiation of infection and the appearance of clinical signs. This would explain the absence of macrophages in previous histopathologic studies on human corneal specimens10 and their detection in experimental animals.5-7 It should be emphasized that the current results, as well as previous findings, indicate that the normal conjunctival macrophage population does not totally inhibit corneal infection in all animals but appears to limit the severity and chronicity of corneal disease. This effect may be exerted shortly after the parasites bind to the corneal epithelial surface and, thus, would not be detected readily by conventional histopathologic methods.

The possibility that other phagocytic cells, such as neutrophils, influence the incidence and severity of...
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FIGURE 5. Photomicrograph of a cornea from a Chinese hamster treated by subconjunctival injections of C12MDP-LIP. Eye was removed 7 days after parasite challenge. Note the extensive stromal swelling. Bar = 20 μm. LIP = liposome.

The influence of C12MDP-LIP on Langerhans cells, another potentially important cell in the protection against Acanthamoeba keratitis, has not been examined. However, Langerhans cells have only limited phagocytic activity and would not be expected to ingest liposomes. Moreover, previous studies have

FIGURE 6. Photomicrograph of a cornea from a Chinese hamster treated by subconjunctival injections of PBS-LIP. Eye was removed 7 days after parasite challenge. Bar = 20 μm. PBS = phosphate-buffered saline; LPS = liposome.

FIGURE 7. Photomicrograph of a cornea from a Chinese hamster treated by subconjunctival injections of PBS-LIP. Eye was removed 14 days after parasite challenge. Bar = 20 μm. PBS = phosphate-buffered saline; LPS = liposome.

Acanthamoeba keratitis was not considered. We have been unable to eliminate neutrophils specifically without influencing other types of cells in the process. However, we suspect that neutrophils present in corneal lesions appear in response to necrosis, just as they do in other infectious diseases and pathologic conditions.

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FIGURE 8. Photomicrograph of a cornea from a Chinese hamster treated by subconjunctival injections of C12MDP-LIP. Eye was removed 14 days after parasite challenge. Note persistent stromal edema. Neutrophils (arrowhead), plasma cells (open arrow), and occasional macrophages (solid arrow) were present in the stroma. Bar = 20 μm. LIP = liposome.
shown that dendritic cells, such as Langerhans cells, are not affected by C12MDP-LIP.

In contrast with other infections of the cornea, such as herpes keratitis, in which the immune response contributes to pathogenesis, it is clear that the presence of immunocompetent cells, such as Langerhans cells, is associated with resistance to *Acanthamoeba* keratitis. Since the development of this method for macrophage depletion, a number of articles have been published on the effect of macrophage depletion on the initiation of immune reactions in a variety of tissues and in the etiology of several diseases. In contrast with our findings, most articles describe a decrease in the severity or course of the disease after clodronate treatment, which is understandable because, in most cases, the diseases studied were immune mediated (e.g., there was allergic encephalomyelitis and autoimmune uveitis). Although this technique might be promising for future clinical use in these immune-mediated diseases and in the prevention of corneal transplant rejection, as described elsewhere, one should take into consideration that macrophage depletion could also cripple a useful defense mechanism against such pathogens as *Acanthamoeba* and *Pseudomonas*.

**Key Words**

*Acanthamoeba* keratitis, Chinese hamster, clodronate, macrophages, liposomes

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**References**


