Macrophage Inflammatory Protein-2 Is a Mediator of Polymorphonuclear Neutrophil Influx in Ocular Bacterial Infection

Karen A. Kernacki, Ronald P. Barrett, Jeffery A. Hobden and Linda D. Hazlett

*J Immunol* 2000; 164:1037-1045; doi: 10.4049/jimmunol.164.2.1037

http://www.jimmunol.org/content/164/2/1037

---

**References**  
This article cites 35 articles, 22 of which you can access for free at:  
http://www.jimmunol.org/content/164/2/1037.full#ref-list-1

**Subscription**  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

**Permissions**  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852  
Copyright © 2000 by The American Association of Immunologists. All rights reserved.  
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Macrophage Inflammatory Protein-2 Is a Mediator of Polymorphonuclear Neutrophil Influx in Ocular Bacterial Infection

Karen A. Kernacki,* Ronald P. Barrett,* Jeffery A. Hobden,† and Linda D. Hazlett2**

Polymorphonuclear neutrophils (PMN) in Pseudomonas aeruginosa-infected cornea are required to clear bacteria from affected tissue, yet their persistence may contribute to irreversible tissue destruction. This study examined the role of C-X-C chemokines in PMN infiltration into P. aeruginosa-infected cornea and the contribution of these mediators to disease pathology. After P. aeruginosa challenge, corneal PMN number and macrophage inflammatory protein-2 (MIP-2) and KC levels were compared in mice that are susceptible (cornea perforates) or resistant (cornea heals) to P. aeruginosa infection. While corneal PMN myeloperoxidase activity (indicator of PMN number) was similar in both groups of mice at 1 and 3 days postinfection, by 5–7 days postinfection corneas of susceptible mice contained a significantly greater number of inflammatory cells. Corneal MIP-2, but not KC, levels correlated with persistence of PMN in the cornea of susceptible mice. To test the biological relevance of these data, resistant mice were treated systemically with rMIP-2. This treatment resulted in increased corneal PMN number and significantly exacerbated corneal disease. Conversely, administration of neutralizing MIP-2 pAb to susceptible mice reduced both PMN infiltration and corneal destruction. Collectively, these findings support an important role for MIP-2 in recruitment of PMN to P. aeruginosa-infected cornea. These data also strongly suggest that a timely down-regulation of the host inflammatory response is critical for resolution of infection. The Journal of Immunology, 2000, 164: 1037–1045.

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that is responsible for causing sight-threatening corneal infections (1). Both host-derived and bacterial factors (e.g., exoproteases and exotoxins) are thought to contribute to the rapidly progressing liquefactive stromal necrosis observed during infection (2–6). Previous studies have suggested that the rapid recruitment of polymorphonuclear neutrophils (PMN)3 to the inflamed cornea after infection is essential to control bacterial replication, but their persistence may also be associated with extensive stromal damage and subsequent scarring or perforation (2, 7–9). The infiltration of PMN into inflamed tissue is controlled largely by the local production of various proinflammatory mediators. In the mouse, two members of the C-X-C family of chemokines, macrophage inflammatory protein (MIP)-2 and KC, are thought to be functional homologues of human IL-8. Similar to IL-8, MIP-2 and KC are potent chemoattractants and activators of PMN (10). Various in vivo models of inflammation have demonstrated direct correlation of PMN influx into tissue with the presence of MIP-2 and/or KC (11–17). In relation to corneal disease, recent studies have demonstrated that MIP-2, but not KC, is the major chemokine that attracts PMN into HSV-1-infected corneas (18, 19).

Our working hypothesis is that the final outcome following P. aeruginosa corneal infection (restoration of corneal clarity vs corneal perforation) depends upon the host’s ability to rapidly remove the invading pathogen. Following bacterial clearance, appropriate modulation of the corneal inflammatory response must occur to allow wound healing. We propose that mice that are unable to efficiently regulate the inflammatory response in cornea will undergo permanent tissue damage following ocular bacterial challenge. To test this, inbred strains of mice that are either susceptible or resistant to corneal infection were examined for corneal PMN number, bacterial load, and for MIP-2 and KC mRNA and protein expression after P. aeruginosa corneal challenge. In addition, recombinant murine chemokine was administered to resistant mice after infection to determine whether this treatment exacerbated disease pathology. Lastly, a neutralizing anti-chemokine polyclonal Ab (pAb) was given to susceptible mice after infection to test whether corneal stromal damage could be reduced.

Materials and Methods

Infection of mice

Young adult (8 wk) BALB/cByJ (BALB/c) (resistant) and C57BL/6J (B6) (susceptible) mice (The Jackson Laboratory, Bar Harbor, ME) were used for these studies. Before corneal infection, mice were lightly anesthetized with isoflurane (Aerrane; Anaquest, Madison, WI) and placed beneath a stereoscopic microscope at ×40 magnification. The central cornea of the left eye was scarified with three 1-mm incisions using a sterile 25 5/8-gauge needle. Random eyes were routinely examined histologically to ensure that the wounds penetrated only the epithelial basal lamina and superficial corneal stroma. A bacterial suspension (5 μl) containing 1.0 × 105 CFU of P. aeruginosa strain 19660 (American Type Culture Collection, Manassas, VA), prepared as described previously (20), was topically applied onto the wounded cornea. Eyes were examined macroscopically 24 h postinfection (p.i.) and/or at times described below to ensure that all mice were similarly infected and to monitor the course of disease in the infected mice. All animals were treated humanely and in full compliance with the Association for Research in Vision and Ophthalmology resolution on usage and treatment of animals in research.
Ocular response to infection

Following *P. aeruginosa* corneal infection, ocular disease was graded using the following established scale (21): 0, clear or slight opacity partially covering the pupil; +1, slight opacity fully covering the entire anterior segment; +2, dense opacity partially or fully covering the pupil; +3, dense opacity covering the entire anterior segment; and +4, corneal perforation. To observe eyes whose lids were sealed, mice were anesthetized with isoflurane and sterile PBS was applied to the lids to permit their careful partial opening without inducing corneal perforation. Five mice from each experimental group (BALB/c vs B6, rMIP-2 vs vehicle-treated, or MIP-2 pAb vs NRS-treated) were examined at each time point. A mean clinical score was calculated for each group of mice to express disease severity. This was determined by summation of the scores for each group divided by the total number of mice scored at each time point (22).

Histopathology

For histopathological analysis, whole eyes were enucleated from three mice from each experimental group (BALB/c vs B6, rMIP-2 vs vehicle, and MIP-2 pAb vs NRS-treated) at various selected times after infection. For the comparison of histopathology between BALB/c vs B6 mice, eyes were collected before and at 16 h, and 3, 5, and 7 days p.i. For determination of the effect of MIP-2 treatment, eyes were collected from rMIP-2- and vehicle-treated mice at 3 and 5 days p.i. For the MIP-2 neutralization studies, eyes were collected from MIP-2 pAb- and NRS-treated mice at 6 days p.i. Eyes were enucleated and immersed immediately in PBS, rinsed, and placed in a fixative containing 1% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M Sorenson’s phosphate buffer, pH 7.4 (1:1:1), at 4°C for a total of 3 h. Eyes were transferred into fresh fixative after 1.5 h. Eyes were then dehydrated in graded ethanolys and embedded in Epon-araldite as described previously (7). Thick sections (1.5 μm) were cut, stained with a modified Richardson’s stain, and observed. Representative sections were photographed with a Zeiss Axiohot photomicroscope (Carl Zeiss, Morgan Instruments, Cincinnati, OH) equipped with bright-field optics using Ilford Pan F film (Mobberley, Cheshire, U.K.).

Quantitation of corneal MIP-2 and KC protein levels

MIP-2 and KC protein levels were determined using ELISA kits (R&D Systems, Minneapolis, MN). For these studies, individual corneas were collected from mice before and at 12 h and 1, 3, and 5 days p.i. as described above. Three corneas were collected separately at each time point. Before storage at −70°C, the total weight of each individual cornea was determined. Corneas were then stored in 0.5 ml of serum-free DMEM containing 0.5% Tween 20. Immediately before analysis, samples were thawed and homogenized with a glass Kontes pestle (Fisher, Itasca, IL). Samples were centrifuged at 5,000 × *g* for 10 min, and an aliquot of each supernatant was assayed for MIP-2 and KC protein. Based upon a preliminary ELISA experiment using *P. aeruginosa*-infected corneal tissues, supernatants were diluted 1:10 for MIP-2 and 1:5 for KC in the kit’s assay diluent to permit detection of signal within the linearity of the standard curves for the respective chemokines. The sensitivity of the ELISA was 1.5 pg/ml for MIP-2 and 2.0 pg/ml for KC. Both chemokine ELISA experiments were performed in duplicate to ensure reproducibility of the data. Results are reported as pg chemokine/mg corneal tissue.

*rMIP-2 administration*

Murine rMIP-2 was purchased from R&D Systems (Minneapolis, MN). The lyophilized powder (10 μg) was reconstituted in 2.0 ml of PBS containing 0.1% BSA as suggested by the manufacturer. BALB/c mice (*n* = 5) were anesthetized with Aerrane and then injected i.p. with 0.2 ml (1.0 μg) of rMIP-2 or vehicle (0.2 ml of PBS/0.1% BSA). Time points chosen for administration of rMIP-2 or vehicle were based on the mRNA and protein data presented herein. The rMIP-2 injection studies were performed in duplicate to ensure reproducibility of the data.

*MIP-2 neutralization*

Neutralizing *pAb* to murine MIP-2 (13) was kindly provided by Dr. Nicholas Lukacs (Department of Pathology, University of Michigan, Ann Arbor, MI). For the MIP-2 neutralization studies, B6 mice (*n* = 5) were anesthetized with Aerrane and subsequently injected i.p. with 0.2 ml of neutralizing MIP-2 pAb at 8 h and at 1 and 3 days after *P. aeruginosa* infection. Control mice (*n* = 5) were similarly treated with 0.2 ml of NRS. The neutralization experiments were performed in duplicate to ensure reproducibility of the data.

Statistical analysis

An unpaired, two-tailed Student’s *t* test was used to determine statistical significance for the mean clinical scores, MPO assays, bacterial plate counts, and ELISAs. Mean differences were considered significant at the confidence level of *p* ≤ 0.05.
Results

Ocular response to infection

Ocular disease was graded in BALB/c and B6 mice after corneal challenge to evaluate the progression and outcome of *P. aeruginosa* infection. Fig. 1 shows the mean clinical scores for the two experimental groups from 1 to 7 days p.i. Similar ocular disease grades (1 to 2) were observed in corneas of BALB/c and B6 mice at 1 day p.i. Over the 7 days p.i. examined, BALB/c mice did not progress significantly past a 2 (dense opacity either partially or fully covering the pupil) ocular disease grade. By 7 days p.i., two of five BALB/c mice demonstrated a 1 disease grade (slight opacity covering the anterior segment). By 7 days p.i., perforation was evident in all of the corneas of B6 mice. Significant differences between the BALB/c and B6 mice were found at 3, 5, and 7 days p.i. (p = 0.0353, 0.0003, and 0.0001 at 3, 5, and 7 days p.i., respectively).

Histopathology

Corneal tissue from BALB/c and B6 mice were compared histopathologically for the first week p.i., and these data are presented in Fig. 2. At 16 h p.i., the epithelium was essentially intact in the cornea of resistant BALB/c mice (Fig. 2A). PMN were observed in the central and peripheral corneal stroma and within the anterior chamber. A few PMN were seen in the corneal epithelium, and free bacteria were infrequently seen in the corneal stroma. In susceptible B6 mice, the corneal epithelium was denuded centrally (Fig. 2B), and free bacteria were more frequently observed within the central corneal stroma. Compared with the BALB/c mouse cornea, PMN were rarely seen in the superior one-third of the central corneal stroma of B6 mice. The infiltrating cells in B6 mice were particularly dense in the lower two-thirds of the stroma and in the peripheral cornea. In the latter location, the cellular pattern suggested centripetal migration. (The arrows in Fig. 2B delineate the area of the central cornea.) In B6 mice, PMN also were present in the anterior chamber and within the peripherally intact corneal epithelium. At 3–5 days p.i., the corneal epithelium had thinned in BALB/c mice (data not shown). PMN remained plentiful throughout the cornea and within the anterior chamber. Free bacteria were rarely observed within the corneal stroma. In B6 mice, the epithelium was completely denuded by 3 days p.i. (data not shown). The corneal stroma was centrally thinned (approximately half of normal), and PMN were numerous at the limbus (juncture of cornea and contiguous conjunctiva and sclera) and in the anterior chamber. Compared with BALB/c mice, fewer PMN were seen in the central cornea. By 5 days p.i., corneal stromal thinning remained markedly severe in B6 mice, and numerous PMN were observed throughout the central cornea. Corneal tissue began healing in all

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Ocular disease response in BALB/c and B6 mice. Following *P. aeruginosa* infection, ocular disease grades were averaged at individual times after infection. Results are reported as mean clinical score ± SEM (p = 0.5447, 0.0353, 0.0003, and 0.0001 at 1, 3, 5, and 7 days p.i., respectively).

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Light-microscopic histopathology of BALB/c and B6 corneas following *P. aeruginosa* challenge. *A* and *B* (central corneal region between arrows in *B*) show the central corneal region of BALB/c and B6 mice at 16 h p.i., respectively. *C* and *D* show the central corneal region of BALB/c and B6 mice at 7 days p.i., respectively (*A–D* at ×45 magnification).
BALB/c mice by 5–7 days p.i. Some corneas from these mice were assigned a +1 ocular disease grade by 7 days p.i. (Fig. 1). Corneas from these mice appeared morphologically normal, and only a few PMN were observed within the peripheral and/or central corneal tissue (data not shown). Alternatively, other corneas from BALB/c mice showed a +2 ocular disease grade. Fig. 2C shows the cornea of a BALB/c mouse with a +2 ocular disease grade at 7 days p.i. The epithelium had begun to resurface and PMN, and other inflammatory cells were observed in the corneal stroma. In the stroma, collagen fibers appeared intact and regularly spaced, and PMN were seen in the adjacent anterior chamber. In contrast, by 7 days p.i., corneas from B6 mice had perforated as a result of the extensive stromal collagen destruction (Fig. 2D). No epithelium was present in the central cornea of these mice. PMN and other inflammatory cells remained numerous throughout the cornea and anterior chamber, and the iris was hyperemic.

PMN quantitation in P. aeruginosa-infected corneas

Histopathology results strongly suggested that susceptible vs resistant mice would differ either in kinetics and/or in the number of PMN that infiltrate the cornea after bacterial challenge. Therefore, we next used an established MPO assay to estimate total corneal PMN number in the two experimental groups of mice. No MPO activity was detected in uninfected corneal tissue from either mouse strain (data not shown). Similar amounts of enzyme activity were detected in BALB/c and B6 mouse cornea from 1 to 3 days p.i. (Fig. 3) In BALB/c mice, peak MPO activity was observed at 3 days p.i., whereas MPO levels continued to increase in B6 mice through 7 days p.i. (last time point examined in these studies). Significant differences in MPO activity between BALB/c and B6 mice were detected at both 5 and 7 days p.i. (p = 0.0001 and 0.008 at 5 and 7 days p.i., respectively).

Quantitation of viable bacteria in infected corneal tissue

To determine whether delayed infiltration of PMN into the superior one-third of the central cornea of B6 mice was associated with impaired clearance of bacteria from affected tissue, viable bacteria were quantitated in BALB/c and B6 corneal tissue from 1 to 9 days p.i. The mean log_{10} number of viable bacteria per BALB/c and B6 cornea at each time p.i. is shown in Fig. 4. No difference in bacterial number was observed between resistant and susceptible mice at 1 and 3 days p.i. After 3 days p.i., BALB/c mice began to clear the invading pathogen from corneal tissue. Only 2 log units of bacteria/cornea remained in this experimental group by 9 days p.i. In contrast, reduction of ocular load below 6 log units of bacteria was not observed in B6 mice until 9 days p.i. Significant differences between BALB/c and B6 mice were found from 5 to 9 days p.i. (p = 0.0041, 0.0016, and 0.0023 at 5, 7, and 9 days p.i., respectively).

Quantitation of MIP-2 and KC mRNA levels following P. aeruginosa infection

To determine whether PMN infiltration into P. aeruginosa-infected corneas was correlated with local corneal C-X-C chemokine production, the amount of MIP-2 and KC mRNA was determined in uninfected and infected corneal tissue from BALB/c and B6 mice using RNase protection assays. Data from two separate experiments are shown in Tables I and II. Neither MIP-2, nor KC mRNA transcripts were detected in uninfected corneal tissue. Initially (6 h p.i.), low levels of MIP-2 mRNA were found in the cornea of BALB/c mice, whereas MIP-2 was not detected in the cornea of B6 mice at this time under the assay conditions tested (Table I). Comparable amounts of MIP-2 transcripts were found in corneal tissue of both mouse strains from 12 h to 3 days p.i. A peak

<table>
<thead>
<tr>
<th>Time p.i.</th>
<th>BALB/c</th>
<th>B6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>12 h</td>
<td>123.9</td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>542.6</td>
</tr>
<tr>
<td></td>
<td>3 days</td>
<td>423.9</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>90.0</td>
</tr>
</tbody>
</table>

Expt. 2

<table>
<thead>
<tr>
<th>Time p.i.</th>
<th>BALB/c</th>
<th>B6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>12 h</td>
<td>88.6</td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>335.8</td>
</tr>
<tr>
<td></td>
<td>3 days</td>
<td>323.9</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>61.6</td>
</tr>
</tbody>
</table>

*Five micrograms of total corneal RNA/sample was assayed by RNase protection assay for MIP-2 mRNA. Results are reported as amol MIP-2 mRNA/μg total RNA.
 FIGURE 5. Corneal MIP-2 protein levels in BALB/c and B6 mice before and at 1–5 days p.i. Individual corneas from BALB/c and B6 mice were analyzed for MIP-2 by ELISA before and after *P. aeruginosa* corneal infection. Three corneas were collected from each mouse strain at the individual time points. Results are reported as pg MIP-2/mg corneal tissue ± SEM (p = 0.7865, 0.0021, 0.0049, and 0.0017 at 12 h and 1, 3, and 5 days p.i., respectively).

FIGURE 6. Corneal KC protein levels in BALB/c and B6 mice before and at 1–5 days p.i. Individual corneas from BALB/c and B6 mice were analyzed for KC by ELISA before and after *P. aeruginosa* corneal infection. Three corneas were collected from each mouse strain at the individual time points. Results are reported as pg KC/mg corneal tissue ± SEM (p = 0.2654, 0.013, 0.3652, and 0.4051 at 12 h and 1, 3, and 5 days p.i., respectively).

Table II. *KC mRNA levels in *P. aeruginosa*-infected corneas*

<table>
<thead>
<tr>
<th>Time p.i.</th>
<th>BALB/c</th>
<th>B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>6 h</td>
<td>33.4</td>
<td>24.1</td>
</tr>
<tr>
<td>12 h</td>
<td>153.8</td>
<td>96.3</td>
</tr>
<tr>
<td>1 day</td>
<td>259.5</td>
<td>282.7</td>
</tr>
<tr>
<td>3 days</td>
<td>11.4</td>
<td>19.1</td>
</tr>
<tr>
<td>5 days</td>
<td>1.9</td>
<td>19.4</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>6 h</td>
<td>45.8</td>
<td>37.8</td>
</tr>
<tr>
<td>12 h</td>
<td>85.7</td>
<td>92.1</td>
</tr>
<tr>
<td>1 day</td>
<td>119.0</td>
<td>140.0</td>
</tr>
<tr>
<td>3 days</td>
<td>7.0</td>
<td>10.2</td>
</tr>
<tr>
<td>5 days</td>
<td>4.9</td>
<td>8.2</td>
</tr>
</tbody>
</table>

*p* Five micrograms of total corneal RNA/sample was assayed by RNase protection assay for KC mRNA. Results are reported as amol KC mRNA/μg total RNA.

in the level of MIP-2 transcript expression was found in both mouse strains by 1 day p.i. By 5 days p.i., corneal MIP-2 mRNA levels began to decline in resistant BALB/c mice. Alternatively, the amount of MIP-2 transcripts detected in B6 mouse cornea remained elevated through 5 days p.i. Conversely, the kinetics and amounts of KC mRNA detected in infected BALB/c and B6 corneal tissue were similar at all time points examined (6 h to 5 days p.i.) (Table II). The levels of KC mRNA peaked at 1 day p.i. and declined thereafter in both groups of mice.

Quantitation of MIP-2 and KC protein levels following *P. aeruginosa* infection

In addition to mRNA quantitation, the amount of MIP-2 and KC protein also was determined in uninfected and infected corneal tissue by ELISA. Data from a representative experiment are shown in Figs. 5 and 6. Neither MIP-2 nor KC was detected in uninfected corneal tissue. A low level of MIP-2 was detected in corneal tissue from BALB/c and B6 mice by 12 h p.i. (Fig. 5). The amount of detectable MIP-2 protein peaked at 3 days p.i. in BALB/c mice and slowly declined thereafter. In contrast, the amount of MIP-2 protein in B6 cornea continued to increase through 5 days p.i. (the last time point tested). Significantly greater amounts of MIP-2 protein were detected in B6 vs BALB/c mouse cornea from 1 to 5 days p.i. (p = 0.0021, 0.0049, and 0.0017 at 1, 3, and 5 days p.i., respectively). In contrast to MIP-2, the amount of KC protein was elevated in both groups of mice by 12 h p.i. (Fig. 6). Peak KC levels were detected in B6 and BALB/c mouse cornea at 12 h and 1 day p.i., respectively. Analogous to the KC mRNA data described above, the corneal levels of KC protein declined after 12 h to 1 day p.i. Statistically greater amounts of KC were found only at 1 day p.i. in B6 mice (p = 0.013), whereas no differences in corneal KC protein were found when KC levels were analyzed in a second similar experiment (data not shown).

rMIP-2 administration to resistant mice

The above studies suggested that the ability to restore corneal clarity following *P. aeruginosa* challenge is associated with down-regulation of MIP-2 expression and ultimately reduction in PMN number. Therefore, to ascertain the biological relevance of these data, we next tested whether systemic administration of rMIP-2 exacerbated corneal disease in resistant BALB/c mice. For these studies, BALB/c mice were injected i.p. with 1.0 μg of rMIP-2 (or vehicle for control mice) at 1 and 3 days p.i. Mean clinical scores from rMIP-2- or vehicle-treated mice were calculated and are shown in Fig. 7. Similar ocular disease grades were observed in both experimental groups at 1 day p.i. (before rMIP-2 or vehicle treatment). By 3 days p.i., mice treated with rMIP-2 had significantly higher ocular disease grades (p = 0.049). While vehicle-treated mice did not progress past a +2 ocular disease grade throughout the experiment, the corneal tissue of mice injected systemically with rMIP-2 perforated (+4) by 5–7 days p.i. (p = 0.008 and 0.001 at 5 and 7 days p.i., respectively).

Corneal tissue from mice treated systemically with rMIP-2 or vehicle also were examined histopathologically at 3 and 5 days p.i. At 5 days p.i., the corneal epithelium was centrally denuded and patchy at the periphery in mice treated with rMIP-2. Extensive stromal thinning, marked corneal edema, and numerous PMN were evident in the cornea of these mice. In addition, ingrowth of blood vessels migrating from the limbus into the peripheral cornea was observed (Fig. 8). When compared with vehicle-treated mice, the vessels in the rMIP-2-treated group were of larger luminal diameter, thicker walled, and inflammatory cell cuffing in the adventia...
was present. Similar differences between rMIP-2- and vehicle-treated mice were observed at 3 days p.i. (data not shown). In contrast to rMIP-2-treated mice, ocular disease was significantly reduced in vehicle-treated mice. A cornea with a +1 ocular disease grade at 5 days p.i. is shown in Fig. 8B. At this time p.i., the epithelium was intact from limbus. Compared with corneas from rMIP-2-treated mice, collagen fibrils were intact and regularly spaced and only slight corneal edema was seen in the superficial stroma. Qualitatively fewer PMN were seen throughout the corneal tissue of the vehicle-treated animals.

As treatment of resistant mice with rMIP-2 was qualitatively associated with a greater number of PMN in infected corneal tissue, PMN MPO activity was determined in infected corneal tissue collected from rMIP-2- or vehicle-treated mice at 3 and 5 days p.i. to confirm the above observations (Fig. 9). Mice treated with rMIP-2 vs vehicle demonstrated a significantly greater amount of corneal MPO activity at both of the time points examined ( \( p \leq 0.008 \) and 0.0023 at 3 and 5 days p.i., respectively).

### MIP-2 pAb treatment in susceptible mice

As an alternative approach to test whether MIP-2 plays a significant role in the pathogenesis of *P. aeruginosa* ocular disease, susceptible B6 mice were treated with multiple injections of neutralizing MIP-2 pAb or NRS after corneal infection. Ocular disease was graded in MIP-2 pAb- and NRS-treated mice from 1 to 9 days p.i. (Fig. 10). Comparable ocular disease grades were observed in mice treated with MIP-2 pAb and NRS from 1 to 5 days p.i. By 7 days p.i., ocular disease was graded significantly higher in MIP-2 pAb treated mice compared to NRS treated mice ( \( p = 0.3466, 0.1411, 0.195, 0.003, \) and 0.001 at 1, 3, 5, 7, and 9 days p.i., respectively).

---

**FIGURE 7.** Ocular disease response in rMIP-2- and vehicle-treated mice. Following *P. aeruginosa* infection, BALB/c mice were treated systemically with either rMIP-2 or vehicle. Ocular disease grades were averaged at individual times after infection. Results are reported as mean clinical score ± SEM ( \( p = 0.3466, 0.049, 0.008, \) and 0.001 at 1, 3, 5, and 7 days p.i., respectively).

**FIGURE 8.** Light microscopic histopathology in BALB/c mice treated with rMIP-2 or vehicle. BALB/c mice were treated with rMIP-2 or vehicle at 1 and 3 days after *P. aeruginosa* corneal infection. Whole eyes were collected for histopathology at 5 days p.i. (A, peripheral corneal region of rMIP-2-treated mouse; B, peripheral corneal region of vehicle-treated mouse) (A and B at ×440 magnification). Arrows in A and B show blood vessel ingrowth into the peripheral cornea.

**FIGURE 9.** Corneal MPO activity in rMIP-2- vs vehicle-treated mice at 3 and 5 days p.i. Following *P. aeruginosa* infection, BALB/c mice were treated systemically with either rMIP-2 or vehicle. Individual corneas ( \( n = 3 \) ) were collected from rMIP-2- and vehicle-treated mice at 3 and 5 days p.i. and analyzed for MPO activity. Results are reported as units MPO/cornea ± SEM ( \( p = 0.008 \) and 0.0023 at 3 and 5 days p.i., respectively).

**FIGURE 10.** Ocular disease response in MIP-2 pAb-and NRS-treated mice. B6 mice were treated i.p. with either MIP-2 pAb or NRS before and after *P. aeruginosa* corneal challenge. Ocular disease grades were averaged at individual times after infection. Results are reported as mean clinical score ± SEM ( \( p = 0.3466, 0.1411, 0.195, 0.003, \) and 0.001 at 1, 3, 5, 7, and 9 days p.i., respectively).
days p.i., all of the corneas in the NRS-treated group had perforated (+4 ocular disease grade). Four of five B6 mice treated with MIP-2 pAb displayed +2 ocular disease grades up to 9 days p.i. (termination of experiment) (p < 0.003 and 0.001 at 7 and 9 days p.i., respectively).

Fig. 11 shows the corneal histopathology in MIP-2 pAb- and NRS-treated B6 mice at 6 days p.i. (the time point where corneal perforation was first observed in NRS-treated mice). In MIP-2 pAb-treated mice, the corneal epithelium was slightly irregular and intact collagenous fibers were observed throughout the superficial and deep stromal regions of the cornea. Moreover, corneal swelling was modest in these mice. In contrast, the epithelium was denuded and/or patchy in the NRS-treated group. Likewise, this group of mice demonstrated extensive degradation of the corneal stroma with total dissolution of stromal fibrillar collagen bundles along with pronounced corneal edema. Numerous PMN were present throughout the cornea and in the anterior chamber of the NRS-treated mice. Qualitatively, corneal PMN appeared to be fewer in number in MIP-2 pAb-treated vs NRS-treated mice.

When MPO activity was measured in corneas of MIP-2 pAb- and NRS-treated mice, significantly less enzyme activity was found in the corneal tissue from mice treated with MIP-2 pAb (p = 0.039, 0.002, and 0.001 at 3, 5, and 7 days p.i., respectively) (Fig. 12). Alternatively, treatment of mice with MIP-2 pAb did not affect corneal bacterial load. The number of viable bacteria isolated from MIP-2 pAb- and NRS-treated mice was similar between 3 and 7 days p.i. (Fig. 13).

Discussion

P. aeruginosa-induced keratitis is a potentially devastating ocular inflammatory disease characterized by rapidly progressing liquefactive stromal necrosis that often leads to corneal perforation within 24–48 h (1). Despite rapid sterilization of ocular tissues achieved by antibiotic therapy, the symptoms of the infection have been reported to persist for days or even weeks suggesting that host-inflammatory processes (e.g., PMN) and/or residual bacterial products (e.g., LPS or exoproteins) induce much of the observed tissue destruction (9). In our model of corneal infectious disease, the hypothesis currently tested is that timely down-regulation of the host inflammatory response after corneal P. aeruginosa challenge promotes restoration of corneal clarity and ocular integrity. In contrast, persistence or continued up-regulation of this response leads to irreversible host-induced corneal tissue damage and perforation.
For the current studies, progression of *P. aeruginosa*-induced ocular disease was monitored in BALB/c and B6 mice from 1 to 7 days p.i. (Fig. 1). Previous studies demonstrated that both BALB/c and B6 mice were susceptible to corneal challenge when the infecting dose of *P. aeruginosa* strain 19660 (American Type Culture Collection) was 10^6 CFU (30). In the studies described herein, mice were infected with 10^6 CFU (20). Using 10^6 CFU, all B6 mice displayed corneal perforation by 7 days p.i. In contrast, by 7 days p.i., some variation in the degree of corneal opacity (reflected by +1 to +2 ocular disease grades) was observed in BALB/c mice. Nonetheless, it was apparent that the cornea from these mice began to heal by 5–7 d p.i. Histopathological examination of infected corneal tissue at 5–7 days p.i. (Fig. 2) confirmed that corneal healing had begun in BALB/c and that extensive stromal loss and perforation had occurred in B6 mice.

The histopathology also suggested that differences in the number and/or kinetics of PMN existed between resistant (cornea heals) and susceptible (cornea perforates) mice. Well-defined differences in the spatial distribution of PMN in cornea were observed between BALB/c (PMN distributed throughout the entire cornea) and B6 (superior central cornea essentially devoid of PMN) mice from 16 h and 3 days p.i. (Fig. 2, A and B and data not shown). Furthermore, an overall greater number of PMN were observed in the cornea from B6 vs BALB/c mice at 5 and 7 days p.i. Use of an established PMN MPO assay to quantitate total corneal PMN number provided verification that B6 mice had a significantly greater number of PMN in corneal tissue, but only at later times p.i. (Fig. 3). In both BALB/c and B6 mice, PMN number correlated well with the degree of corneal opacity and corneal integrity. In this regard, those mice displaying +1 vs +2 ocular disease grades had fewer corneal PMN and reduced ocular disease when compared with those mice with a +3 ocular disease grade.

When bacterial load was quantitated in the cornea of BALB/c and B6 mice p.i., a significantly greater number of viable bacteria were detected in the cornea of B6 vs BALB/c mice from 5 to 9 days p.i. (Fig. 4). Two- to 3-log fold differences in viable bacterial number were found between resistant and susceptible mice at 7 and 9 days p.i., respectively. These data suggested that there may be a functional defect in the ability of PMN from B6 mice to kill *P. aeruginosa*. To address this issue, we used established PMN phagocytic and microbicidal assays (31) to test the functional integrity of peripheral blood-derived PMN from B6 vs BALB/c mice. These experiments showed that there was no difference in the ability of PMN from either mouse strain to phagocytose and kill *P. aeruginosa* (data not shown), hence ruling out a defect in PMN as the cause for persistence of bacteria in the cornea of B6 mice. Alternatively, the histopathology data presented herein suggested that delayed infiltration of PMN into the superior central cornea of B6 mice may alter their ability to remove bacteria from the affected tissue at later times p.i. (Fig. 2). We hypothesize that this delay could allow for continued replication of bacteria in B6 corneal tissue, as reflected by the observed increase in bacterial load at later times p.i. We propose that a greater number of PMN would subsequently be required to ultimately reduce the bacterial load. Consequently, this increase and/or persistence of PMN could directly contribute to the outcome of extensive stromal destruction observed in B6 mice.

In susceptible B6 mice, aberrant regulation of the inductive and/or resolution phases of the host-inflammatory response also could contribute to the susceptible phenotype. In this regard, differential regulation of the expression and secretion of one or more soluble proinflammatory mediators may contribute to the disparate PMN response in susceptible vs resistant mice. To test this, the expression of two C-X-C chemokines (KC and MIP-2) was examined in corneal tissue of BALB/c and B6 mice after *P. aeruginosa* challenge. Similar amounts of KC mRNA and protein were found in the cornea of both strains of mice from 12 h to 5 days after infection (Fig. 6 and Table II). The early peak of KC expression (12 h to 1 day p.i.) correlated with the initial infiltration of PMN into infected cornea, implying that KC could be involved in the early corneal PMN response. However, the rapid decrease of KC expression in B6 cornea by 3 days p.i. strongly suggests that this chemokine is not critical for the persistence of PMN in the cornea of susceptible mice.

In contrast to KC, MIP-2 levels (mRNA and protein) in cornea of susceptible mice were associated with increased corneal PMN at later times p.i. This increase correlated with the onset of corneal ulceration and perforation (Figs. 1 and 5 and Table I). Alternatively, timely down-regulation of corneal MIP-2 levels and PMN number in resistant mice was followed by corneal healing and re-establishment of ocular integrity. One potential mechanism by which MIP-2 could amplify the local PMN inflammatory response has been proposed by both Gainet et al. and Mercer-Jones et al. (32, 33). Their studies showed that activated PMN produce MIP-2 (mouse) or IL-8 (human) and that production of the respective chemokine by PMN produces an autoamplification loop of PMN recruitment and activation at inflamed sites. It is possible that such augmentation of the cyclic MIP-2/PMN response in B6 mice could lead to eventual corneal ulceration and perforation. Furthermore, data reported by Yan et al. (19) support the conclusions drawn in the current study regarding MIP-2 and KC. This group showed previously that treatment of mice with neutralizing pAb to MIP-2, but not KC, significantly reduced HSV-induced corneal inflammation and PMN responses.

Because persistence of MIP-2 expression in B6 mice appeared to be associated with continued PMN infiltration and corneal perforation, we next tested whether the resistance phenotype of BALB/c mice could be altered by administration of rMIP-2. For these studies, systemic rMIP-2 administration was used in an attempt to alter disease outcome. This route of injection was selected because others previously have shown that serum proteins can readily extravasate into corneal tissue following induction of a corneal inflammatory response (34, 35). This is due largely to the ingrowth from the limbus of blood vessels into the normally avascular cornea. As vessels invade inward, it is feasible to hypothesize that a serum-derived local chemokine gradient (from the systemically administered rMIP-2) could be established within the cornea that could contribute to the recruitment of PMN. The rMIP-2 itself also may accentuate the angiogenic response in cornea as described in studies reported by Keane et al. (36). The data in Fig. 8 show that systemic administration of rMIP-2 vs vehicle increased the size and frequency of blood vessels infiltrating the peripheral cornea. In addition, there was an increase in the number of inflammatory cells surrounding and within the lumen of these vessels following rMIP-2 treatment. Thus, the data presented in the current study provide structural evidence of the mechanism by which a local chemokine gradient could be established in the cornea of rMIP-2-treated mice.

Measurement of corneal MPO activity in rMIP-2- vs vehicle-treated mice at 3 and 5 days p.i. confirmed that the systemic treatment protocol described above increased the total number of PMN in cornea of rMIP-2-treated mice (Fig. 9). Examination of corneal histopathology in the two experimental groups of mice also showed that mice treated with MIP-2 had exacerbated corneal disease (Fig. 8). In combination, these studies demonstrate that MIP-2 can regulate the PMN response in *P. aeruginosa*-infected cornea and that an increase in the number of this cell type correlates with extensive stromal tissue destruction.
As an additional test of the in vivo biological role of MIP-2 in PMN recruitment and corneal destruction, susceptible B6 mice were treated with a neutralizing MIP-2 pAb or NRS after corneal challenge. Treatment with MIP-2 pAb vs NRS was effective in reducing corneal PMN number, stromal destruction, and perforation in B6 mice up to 9 days p.i. without an increase in bacterial load (Figs. 10–13). Because a large number of bacteria remained in the cornea of MIP-2 pAb-treated mice, it is unreasonable to assume that complete protection (e.g., total corneal healing) against *P. aeruginosa*-induced stromal destruction and perforation was achieved using pAb treatment alone. It may be necessary to combine MIP-2 pAb with antibiotics to reduce both the host response and bacterial load to increase the likelihood of corneal healing after bacterial challenge.

In summary, our results directly demonstrate that MIP-2 is a mediator of corneal PMN infiltration. These studies suggest that prolonged expression of this chemokine in *P. aeruginosa*-infected cornea contributes to persistence of this cell in an infected B6 cornea and ultimately to corneal ulceration and perforation. In contrast, resistant BALB/c mice resolve corneal infection without extensive stromal destruction, and this correlates with their ability to eliminate the invading pathogen by attraction of a sufficient number of PMN to the site of infection. Following bacterial clearance, restoration of corneal clarity and ocular integrity in resistant mice is associated with cessation of MIP-2 expression and subsequent down-regulation of PMN migration into the cornea.

References