Aging and PMN Response to P. aeruginosa Infection

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PURPOSE. Alterations in immune system function associated with aging may contribute to increased morbidity in this population of individuals. The current studies were performed to determine aging-related changes in polymorphonuclear neutrophil (PMN) function after corneal infection with Pseudomonas aeruginosa.

METHODS. Total PMN number, macrophage inflammatory protein (MIP)-2 mRNA and protein expression, and ocular bacterial load were determined in 8-week- and 12-month-old inbred BALB/c mice at various times after infection with P. aeruginosa. In addition, 12-month-old mice were treated systemically with the MIP-2 polyclonal antibody (pAb) to determine the effects of MIP-2 neutralization on ocular disease and PMN recruitment.

RESULTS. Histologically, PMN infiltration into the cornea of 12-month-old mice was delayed initially and was associated with an inability to reduce bacterial load at later postinfection (PI) times. In addition, a significantly greater number of PMNs were found in the cornea of 12-month-old mice at later PI times. The increase in PMN number in 12-month-old mice correlated with a persistence of MIP-2 expression in cornea at these later times. Systemic treatment of 12-month-old mice with neutralizing MIP-2 pAb versus normal rabbit serum (NRS) resulted in reduced corneal PMN number and ocular disease.

CONCLUSIONS. These data provide evidence that persistence of PMN in the cornea of 12-month-old mice contributes to corneal tissue destruction after P. aeruginosa challenge. Further evidence also is provided that the chemoattractant MIP-2 pAb offers protection and reduces the altered PMN response in these animals.

Alterations in immune system function associated with aging result in increased susceptibility to infection. Both humoral and cell-mediated immune responses to various pathogens are affected by this process.1 The aging process also has been associated with changes in the functional capacity of cells involved in early, nonspecific immunity.2,3 Of these, changes in PMN function have been the best characterized. These include an age-related decline in PMN recruitment to sites of inflammation and defects in the phagocytosis and killing of microbial pathogens. In addition to showing alterations in immune cell effector function, previous studies have demonstrated that expression and release of soluble mediators of inflammation (e.g., cytokines and chemokines) are poorly regulated in aged individuals.4–6 Several of these studies suggested that the aging process was associated with a decreased ability to downregulate excessive inflammatory mediator production after bacterial infection or lipopolysaccharide (LPS) challenge. Although various aging models of infectious disease have been described to support this hypothesis, little is known about the role of the host response in the pathogenesis of ocular infection in the aged. In clinical studies, predisposing factors for corneal infections in the elderly (62–65 years old) have been reported and include concomitant eye or systemic (diabetes, dementia, or chronic alcoholism) disease. These patients were also found to be at high risk for infection with Pseudomonas aeruginosa as well as other ocular pathogens.7,8

Previous experimental data from this laboratory using a young (8-week-old) versus aged (12-month-old) outbred mouse model suggest that susceptibility to P. aeruginosa challenge involves defects in inflammatory mediator expression and/or PMN function.9–13 The current studies were performed to elucidate which specific host factors (e.g., cytokines and/or chemokines) contribute to the extensive stromal destruction and corneal perforation in 12-month-old animals.

MATERIALS AND METHODS

Infection of Mice

Young adult (8 weeks) and aged (12 months) BALB/cByJ (BALB/c) mice (Charles River, Kingston, NY) were used for the current studies. Before corneal infection, mice were 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. A bacterial suspension (5 μl) containing 1.0 × 106 colony-forming units (CFU) of P. aeruginosa (American Type Culture Collection strain 19660), prepared as described before,14 was topicaly applied onto the wounded cornea. All animals were treated humanely and in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
Ocular Response to Infection

After *P. aeruginosa* corneal infection, ocular disease was graded using the following established scale: 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. Five mice from each experimental group were examined from postinfection days (DPI) 1 to 7. The experiment was repeated once similarly, and representative data are shown.

Histopathology

For histopathologic analysis, whole eyes were enucleated from three mice from each experimental group (8-week- versus 12-month-old BALB/c mice) at 1 and 5 DPI. Eyes were enucleated and immersed immediately in phosphate-buffered saline (PBS), rinsed, and placed in a fixative containing 1% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M Sörensen’s phosphate buffer (pH 7.4; 1:1:1) at 4°C for a total of 3 hours. Eyes were then dehydrated in graded ethanols and embedded in Epon-Araldit, as described previously. Thick sections (1.5 μm) of the eye were cut from each group of three mice, stained with a modified Richardson’s stain, and observed. Representative sections were photographed with a photomicroscope (Axiopt; Carl Zeiss, Thornwood, NY) equipped with bright field optics and film (pan F film; Ilford, Cheshire, UK).

Quantitation of PMN in Corneal Tissues

A myeloperoxidase (MPO) assay was used to quantitate the total number of PMN in cornea after infection. At selected times after infection, three corneas from each experimental group were collected for analysis. After collection, individual corneas were immersed in 1.0 ml of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. Samples were sonicated for 10 seconds on ice and subsequently freeze thawed three times. After centrifugation, an aliquot of the supernatant (0.1 ml) was added to 2.9 ml of 50 mM phosphate buffer containing o-dianisidine dihydrochloride (16.7 mg/100 ml) and hydrogen peroxide (0.0005%). The change in absorbancy at 460 nm was monitored for 5 minutes, and the slope of the line was determined for each sample. The change in absorbancy at 460 nm was monitored for 5 minutes, and the slope of the line was determined for each sample. Representative results from two similar experiments are reported, and the data expressed as atomoles checmokine mRNA per microgram total RNA.

Quantification of MIP-2 mRNA

RNase protection assays (RPAs) were used to quantify the level of macrophage inflammatory protein (MIP)-2 mRNA in cornea, as described previously. Briefly, corneal tissue was collected from 8-week- and 12-month-old mice before and at various times after infection. Samples were flash frozen in liquid nitrogen and stored at −70°C until extraction of RNA. Four corneas were pooled for an individual sample. Total RNA was extracted from corneal tissue using RNAzol B (Tel-Test; Friendsville, TX). Three micrograms of total RNA from each sample were hybridized overnight at 56°C to 300 pg of 32P-labeled, antisense strand MIP-2 riboprobe. Similarly, various concentrations of unlabeled sense strand MIP-2 standard (7.8–250 pg) were hybridized to the same amount of riboprobe. After hybridization, samples were digested with T1 nuclease (Gibco; Gaithersburg, MD), and nuclease-protected fragments were resolved on a 4.5% urea-containing sequencing gel. Protected bands were observed by exposing the dried gel to x-ray film and quantitated using a densitometer with image analysis software (Molecular Dynamics; Sunnyvale, CA). Representative results from two similar experiments are reported, and the data expressed as atomoles checmokine mRNA per microgram total RNA.

Quantitation of MIP-2 Protein Levels

Corneal MIP-2 protein levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D; Minneapolis, MN), as described previously. For these studies, individual corneas (n = 3/time point) were collected from mice before and at various times after infection. Before storage at −70°C, the total weight of each cornea was determined. Corneas were then stored in 1.0 ml of PBS containing 0.5% Tween 20. Immediately before analysis, samples were thawed and homogenized with a glass pestle (Kontes; Fischer, Itasca, IL). After centrifugation, an aliquot of each supernatant was assayed for MIP-2 protein. Representative results from one of two similar experiments are reported, and the data expressed as picograms MIP-2 per milliliter corneal tissue.

MIP-2 Neutralization

Neutralizing polyclonal antibody (pAb) to murine MIP-2 was kindly provided by Nicholas Lukacs (Department of Pathology, University of Michigan, Ann Arbor). This pAb (1:1000 dilution) has been shown previously to completely neutralize PMN chemotaxis to 30 ng of murine MIP-2 per milliliter. For the MIP-2 neutralization studies, 12-month-old BALB/c mice (n = 5) were anesthetized with isoflurane and injected intraperitoneally with 0.2 ml of neutralizing MIP-2 pAb at 1 day before and at 1 and 3 days after infection. Control mice (n = 5) were similarly treated with 0.2 ml of preimmune, normal rabbit serum (NRS). This experiment was repeated similarly three times, and representative data from one experiment are presented.

Statistical Analysis

An unpaired, two-tailed Student’s *t*-test was used to determine statistical significance for the ocular disease grades, 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

Previous studies have demonstrated that outbred young (8 weeks) and aged (12 month) Swiss-ICR mice differ in restoring...
corneal clarity and ocular integrity after corneal infection with \textit{P. aeruginosa}. The current studies tested further the immune response to infection in the more reproducible inbred young versus aged mouse model. Similar to outbred animals, infected corneas of 8-week-old BALB/c mice began to heal by 7 DPI (data not shown). These animals were assigned 1 (faint opacity covering the anterior segment) or 2 (dense opacity partially or fully covering the pupil) ocular disease grades at this time. Alternatively, the cornea of all 12-month-old BALB/c mice perforated (a 4 ocular disease grade) by 7 DPI (\textit{P} \leq 0.0001).

\textbf{Histopathology}

Corneas of 8-week- and 12-month-old BALB/c mice also were examined histopathologically at 1 and 5 DPI and marked differences in the progression of corneal disease were observed (Fig. 1). At 1 DPI, the epithelium was irregular (patchy or denuded centrally while intact peripherally) in the cornea of 8-week-old mice (Figs. 1A, 1C). Numerous PMN were observed in the central (Fig. 1A) and peripheral (Fig. 1C) cornea, and corneal swelling was modest. In contrast, in 12-month-old mice, PMN were not observed in the central cornea at 1 DPI (Figs. 1B, 1D). Instead, PMN were distributed throughout the entire thickness of the peripheral cornea (Fig. 1D), but centripetally migrating cells were also evident. Similar to 8-week-old mice, the epithelium was patchy or denuded in the central cornea of 12-month-old mice, but remained intact at the periphery. Figure 1E shows the cornea of an 8-week-old mouse assigned a +1 ocular disease grade at 5 DPI. In this cornea, the epithelium had resurfaced almost completely, and collagen fibrils in the stroma appeared intact and regularly spaced. Only a few PMN remained in the cornea of 8-week-old mice at this time. At a similar time in 12-month-old mice, the epithelium was completely stripped in the central cornea (Fig. 1F). Numerous PMN were present throughout the entire thickness of the cornea and in the anterior chamber. Extensive degradation of stromal collagen fibrils was observed and was accompanied by pronounced corneal edema. These findings are consistent with an ocular disease grade of +4 (corneal perforation) in aged animals.

\textbf{PMN Quantitation}

Because the corneal histopathology data strongly suggested that 8-week- versus 12-month-old BALB/c mice might differ in both the kinetics and number of PMN that infiltrate the cornea postinfection, we used an established MPO assay to quantitate corneal PMN number in the two experimental groups of mice from 1 to 5 DPI (Fig. 2). These data demonstrated that despite histologic differences, similar amounts of MPO activity were detected in corneal tissue from 8-week- versus 12-month-old mice at 1 DPI. At later times after infection, significantly greater amounts of MPO activity were found in tissue collected from aged mice (\textit{P} = 0.027 and 0.0018 at 3 and 5 DPI, respectively).

\textbf{Quantitation of Viable Bacteria}

Because the localization of PMN in the central cornea appeared initially delayed after infection in 12-month- versus 8-week-old BALB/c mice, we next tested whether this affected the clearance of \textit{P. aeruginosa} from the cornea. For this study, the number of viable bacteria was quantitated in corneal tissue from 8-week- and 12-month-old mice at various times after infection. Despite the histologic data showing no PMN in central cornea, a similar number of viable bacteria were de-
Detected in corneal tissue from 8-week- and 12-month-old mice at 1 and 3 DPI (Fig. 3). Bacterial load peaked in corneal tissue of 8-week-old mice at 3 DPI (between 7 and 8 log units) and declined to approximately 4 log units by 8 DPI. In contrast, approximately 8 log units of bacteria were found in the cornea of 12-month-old mice through 5 DPI. By 8 DPI only a slight reduction in bacterial load was observed in this experimental group. A significantly greater number of viable bacteria were found in the cornea of 12-month- versus 8-week-old BALB/c mice at 5 and 8 DPI ($P < 0.0385$ and $0.0004$ at 5 and 8 DPI, respectively).

Quantitation of MIP-2 mRNA

These data led us to next test whether the persistence of PMN in the cornea of 12-month-old mice was associated with an inability to downregulate the expression and secretion of the PMN chemoattractant MIP-2. For these experiments, MIP-2 mRNA levels were measured in the cornea of 8-week- and 12-month-old mice at various times after infection. Table 1 shows the levels of MIP-2 mRNA in cornea derived from two experiments. Similar amounts of MIP-2 mRNA were observed in 8-week- and 12-month-old mice at 12 hours after infection. Peak corneal expression of MIP-2 message was observed in both experimental groups at 3 DPI, although at this time 12-month-old mice displayed approximately three times the amount of MIP-2 mRNA. Although expression of MIP-2 message had decreased significantly in 8-week-old mice by 7 DPI, a decline was not observed in the cornea of 12-month-old animals.

Quantitation of MIP-2 Protein

In addition to measuring MIP-2 mRNA expression in $P. aeruginosa$-infected cornea, the amount of MIP-2 protein was quantitated in both experimental groups from 1 to 5 DPI. The data from this experiment are shown in Figure 4. Although there was a slight increase in MIP-2 protein in the cornea of 12-month-old mice at 1 DPI, these data were not significant. The amount of MIP-2 protein peaked in 8-week-old mice at 3 DPI and declined thereafter. In contrast, the amount of MIP-2 protein detected in the cornea of 12-month-old mice continued to increase through 5 DPI (last time point tested). Significantly greater amounts of MIP-2 were found in the cornea of 12-month-old mice at 3 and 5 DPI ($P = 0.0232$ and $0.0001$ at 3 and 5 DPI, respectively).

**MIP-2 pAb Treatment of Aged Mice**

Because the persistence of PMN in the infected cornea of 12-month-old mice correlated well with the prolonged in-

![Figure 3. Bacterial number in the corneas of BALB/c mice aged 8 weeks (young) and 12 months (aged). Total viable bacterial numbers were determined in individual corneas of 8-week- and 12-month-old BALB/c mice at various times after infection. Three corneas per experimental group were collected at each time point. Results are reported as log$_{10}$ number viable bacteria per cornea ± SEM. $P = 0.0679$, 0.085, 0.0385, 0.0004 at 1, 3, 5, and 8 DPI, respectively.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932909/)
increased expression of MIP-2, we next tested whether systemic administration of neutralizing MIP-2 pAb\textsuperscript{20} could downregulate the host PMN-mediated component of infection. Twelve-month-old mice were treated with MIP-2 pAb or NRS (control) before and after corneal infection. Ocular disease grades were determined at 7 DPI, at which time, all 12-month-old mice treated with NRS demonstrated corneal perforation, whereas mice treated with the MIP-2 pAb had +2 or +3 ocular disease grades ($P = 0.004$, data not shown). In the MIP-2 pAb–treated mice with a $+5$ ocular disease grade, some corneal thinning was apparent macroscopically; however, perforation did not occur in these animals as of 7 DPI.

Because disease activity was reduced in MIP-2 pAb–treated 12-month-old mice, corneal MPO activity was measured in the two experimental groups to determine whether an effect of MIP-2 pAb treatment was the reduction of corneal PMN number. These data are shown in Figure 5. A similar amount of MPO activity was detected in the cornea of MIP-2 pAb– and NRS-treated mice at 5 DPI. In contrast, mice treated with MIP-2 pAb versus NRS had significantly less corneal MPO activity at 5 DPI ($P = 0.0347$).

**DISCUSSION**

Aging is associated with an increased susceptibility to *P. aeruginosa* corneal infection.\textsuperscript{7,8,11} Previous studies in our laboratory using outbred mice aged 8-weeks (resistant) versus 12-months (susceptible) showed that the early (up to 48 hours after infection) host response to corneal infection (e.g., PMN and cytokine response) was delayed in 12-month-old animals.\textsuperscript{4,5,13} These experiments also suggested that defects in PMN function (e.g., recruitment and phagocytosis) may contribute to an increased bacterial burden in aged mice that could ultimately lead to corneal perforation.\textsuperscript{10,12} Other more recent studies using genetically susceptible young (8-week-old) mice suggested that persistence of PMN in cornea after *P. aeruginosa* challenge contributes to the observed corneal destruction.\textsuperscript{19} These experiments also show that the PMN chemoattractant MIP-2 is an important mediator of this cellular response. The current studies were designed to further characterize the disparate response to corneal infection in 8-week- and 12-month-old mice. For these studies, ocular bacterial load and corneal inflammatory responses were examined in 8-week- and 12-month-old inbred BALB/c mice, rather than outbred ICR mice, because immune-mediated responses are more reproducibly tested in inbred animals. In addition, these responses were examined from 12 hours to 7 DPI, in contrast to our earlier studies in which host responses were examined from 6 to 48 hours after infection. These later times after infection were examined so that differences in the host response at the time of perforation (5–7 DPI) in 8-week- versus 12-month-old mice could be assessed.

When progression of ocular disease was monitored macroscopically in BALB/c mice, these data showed that 8-week- and 12-month-old inbred mice responded to *P. aeruginosa* challenge similar to outbred animals.\textsuperscript{12} That is, corneal perforation was observed in all 12-month-old mice by 7 DPI, whereas corneal healing had begun in 8-week-old mice. Corneal histopathology confirmed the macroscopic observations that PMN were reduced or absent from the central cornea initially (up to 1 DPI) in 12-month-old mice. Whereas the histopathology data showed marked differences in the distribution of PMN in the cornea of 8-week- versus 12-month-old mice at 1 DPI, the overall total number of PMN, as measured by MPO activity, was unexpectedly similar in both groups. In contrast, by 5 DPI, histopathology and MPO assays confirmed similarly that PMN were distributed throughout the entire cornea and were more numerous, respectively, in the cornea of 12-month- versus 8-week-old mice.

The next series of experiments tested whether this histologically observed absence of PMN in the cornea was associated with impaired clearance of *P. aeruginosa* from the cornea of 12-month-old animals. A similar bacterial load was found in the cornea of 8-week- and 12-month-old mice at 1 and 3 DPI, but as early as 4 DPI, 8-week-old mice exhibited a significant reduction in bacterial number. In contrast, bacterial number did not begin to decline in the cornea of 12-month-old mice until 8 DPI, after corneal perforation had already occurred. From these data, we hypothesize that the early absence of PMNs from the central cornea of 12-month-old mice may allow for continued replication of bacteria in the cornea. This increase in bacterial number theoretically would require a greater number of PMNs to ultimately clear the infection. Both the histopathology and MPO data support this theory. In addition to age-related defects in PMN localization in cornea, other functional defects such as in phagocytosis of *P. aeruginosa* also may contribute to the impaired clearance of bacteria in 12-month-old mice. In this regard, in vitro studies have shown impaired uptake and killing of various pathogens, including *P. aeruginosa*, by PMN isolated from aged individuals or rodents.\textsuperscript{5,21,22}

It is also possible that aberrations in the early synthesis and secretion of inflammatory mediators may account for the initial defective PMN response in 12-month-old animals.\textsuperscript{4,5,13}

**FIGURE 5.** Corneal MPO activity in MIP-2 pAb– or NRS-treated 12-month-old BALB/c mice at 3 and 5 DPI. Individual corneas from MIP-2 pAb– and NRS-treated mice were analyzed for PMN MPO activity after corneal infection with *P. aeruginosa*. Three corneas were collected from each experimental group at the individual time points. Results are reported as units MPO/cornea ± SEM. $P = 0.2896$ and 0.0347 at 3 and 5 DPI, respectively.
Accordingly, we showed previously that a reduction in interleukin (IL)-1β and interferon (IFN)-γ levels during the first 12 hours after infection in outbred 12-month-old mice correlated with the absence of corneal intercellular adhesion molecule (ICAM)-1 upregulation and PMN infiltration. Alternatively, previous studies using 8-week-old inbred mice classified as either susceptible or resistant to corneal *P. aeruginosa* challenge suggested that the early PMN response to infection was not mediated by the PMN chemotactant MIP-2.19 We have provided evidence that this is also the case with 8-week-versus 12-month-old mice, because similar amounts of MIP-2 mRNA and protein were found in the cornea of both experimental groups at 12 hours and 1 DPI.

In contrast to the early response to infection, our previous studies using the 8-week-old genetically susceptible–resistant mouse model system showed that continued increased expression of MIP-2 and interleukin (IL)-1 in the cornea of a susceptible strain of mice at later times after infection was associated with the persistence of PMN and ultimate corneal perforation.19,23 The current studies also show a similar persistence of MIP-2 expression in the cornea of 12-month-old susceptible animals. Increased later expression of MIP-2 in the cornea of 12-month-old mice could be due to the continued presence of an inflammatory stimulus (e.g., *P. aeruginosa*) in the cornea of these mice. Alternatively, studies by Himi et al.4 and Bruunsgaard et al.24 suggest that the observed prolonged inflammatory response in the aged also could be due to an altered regulation in the expression and/or secretion of proinflammatory and antiinflammatory cytokines.

Because the corneal expression of MIP-2 in 12-month-old mice remained significantly elevated at 5 to 7 DPI, we next tested whether this chemokine was biologically relevant to this model of infection. For these studies 12-month-old BALB/c mice were systemically administered either neutralizing MIP-2 pAb or NRS (control) before and after *P. aeruginosa* challenge. Whereas the cornea of all mice treated with NRS perforated at 7 DPI, mice treated with MIP-2 pAb displayed reduced ocular disease (+2 to +3 ocular disease grades) at this time. In addition, significantly fewer PMNs were detected in the cornea of MIP-2 pAb–versus NRS-treated mice at both 3 and 5 DPI. These studies indicated that an increase in and/or persistence of local MIP-2 expression in the cornea of 12-month-old mice is one mechanism by which continued PMN recruitment to the inflamed cornea occurred. The increased number or extended persistence of this cell type in cornea has been implicated previously as a major contributor to irreversible stromal damage.16,19 Although perforation was not observed in MIP-2 pAb–treated mice, the cornea of these animals was not normal. Thus, it is likely that other inflammatory mediators participate in the recruitment of PMN to the cornea. In this regard, we tested and found significantly higher amounts of the pleiotropic proinflammatory cytokine IL-1β in the infected cornea of 12-month-versus 8-week-old inbred mice at later times after infection (Hazlett et al., unpublished data, 2000).

In summary, the present study provides evidence that, in 12-month-old mice, aberrant regulation of the host response to corneal challenge with *P. aeruginosa* contributes to corneal stromal destruction and perforation. The histopathology studies together with bacterial load data suggest that early absence of PMNs from the central cornea of 12-month-old mice after infection may occur because in these animals *P. aeruginosa* is not efficiently removed from the cornea. In addition, these studies show that the chemokine MIP-2 (and possibly the cytokine IL-1) contribute to the susceptible phenotype by enhancing recruitment of PMN to the cornea. Because activated PMN secrete various proteolytic enzymes and oxidative metabolites,26 the increased number and persistence of this cell type in 12-month-old animals leads to degradation of various matrix components of the cornea and eventually to corneal perforation.

**References**


