Proinflammatory cytokine deficiency and pathogenesis of Pseudomonas aeruginosa keratitis in aged mice.

J A Hobden, S A Masinick, R P Barrett and L D Hazlett

Proinflammatory Cytokine Deficiency and Pathogenesis of *Pseudomonas aeruginosa* Keratitis in Aged Mice

JEFFERY A. HOBDEN, SHARON A. MASINICK, RONALD P. BARRETT, AND LINDA D. HAZLETT*

Department of Anatomy/Cell Biology, Wayne State University School of Medicine, Detroit, Michigan 48201

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Corneal clarity in young adult Swiss (HSD:ICR) mice is restored after *Pseudomonas aeruginosa* infection. Previous data showed that this response involves a rapid up-regulation of constitutive intercellular cell adhesion molecule-1 (ICAM-1) and migration of inflammatory cells into the cornea. In contrast, in aged mice, there is no up-regulation of corneal ICAM-1, inflammatory cell infiltration into the cornea is delayed, and the cornea perforates. Therefore, the aim of this study was to test whether specific cytokines which up-regulate ICAM-1 expression differ in young and aged mice. Corneas of young (6- to 8-week-old) and aged (1- to 2-year-old) mice were scarified and inoculated with *P. aeruginosa*. The eyes were graded for pathologic changes (score 0 to +4); at 6, 12, 24, and 48 h postinfection (p.i.), six mice from each age group were sacrificed. Three corneas from each group were used for quantitation of interleukin-1β (IL-1β), tumor necrosis factor alpha, and gamma interferon (IFN-γ) by enzyme-linked immunosorbent assay. The remaining three corneas from each age group were harvested for quantitation of viable bacteria by direct plate count determination and for infiltrating polymorphonuclear leukocytes (PMNs) by a myeloperoxidase (MPO) assay. Compared to those of young mice, the corneas of infected aged mice had less IL-1β at 6 h p.i. (P ≤ 0.04) and less IFN-γ at 12 to 48 h p.i. (P ≤ 0.05). Also, compared to those of young mice, corneas of aged mice had fewer PMNs (P ≤ 0.008) by the MPO assay at 6 h p.i. and more viable bacteria (P ≤ 0.01) per cornea by plate count determination at 24 h p.i. These data suggest that the lack of up-regulation of ocular ICAM-1 in aged mice may reflect a reduction in both IL-1β and IFN-γ levels in the infected cornea. Consequently, a sufficient number of PMNs and other inflammatory cells fail to rapidly migrate into the infected corneas of aged mice, the bacterial load is initially greater than that in young mice, and the cornea perforates.

*P. aeruginosa* is a common cause of corneal infections (29). Bacterial exoprotoexases and exotoxins (23, 35) and the host inflammatory response, primarily an influx of polymorphonuclear leukocytes (PMNs) (17, 25), contribute to the rapid liquefactive stromal necrosis characteristic of these infections. Although PMNs may contribute to corneal tissue damage (11, 42), several studies (2, 13, 14, 18, 21) suggest that these cells are essential for eliminating viable bacteria from ocular tissue and restoring corneal clarity. Previously, we observed that corneal clarity in young out-bred Swiss mice (6 to 8 weeks old) is restored after pseudomonal infection whereas corneas of aged mice (1 to 2 years old) perforate (14). Further study showed that, in contrast to young mice, aged animals do not up-regulate intercellular cell adhesion molecule-1 (ICAM-1) above constitutively expressed levels following infection (22). Since ICAM-1 is required for efficient PMN extravasation from the vascular to the tissues, we hypothesized that a deficiency in cytokines known to up-regulate ICAM-1 and migration of inflammatory cells into the cornea. In contrast, in aged mice, expressed levels delays PMN migration into infected corneas, resulting in greater bacterial growth in tissues and ultimately in a poor prognosis. To test the second hypothesis, PMN infiltration into *P. aeruginosa*-infected corneas of aged and young mice was determined at various times postinfection (p.i.) by a myeloperoxidase (MPO) assay and the number of viable bacteria within these corneas was also quantitated.

**MATERIALS AND METHODS**

**Mice.** Female Swiss ICR mice (HSD:ICR) were purchased as young adults (6 to 8 weeks of age) or retired breeders (6 to 9 months of age) from Harlan Sprague-Dawley, Inc. (Indianapolis, Ind.). The mice were housed in clear plastic cages and fed laboratory rodent diet (PMI Feeds, Inc., St. Louis, Mo.) and acidified water ad libitum. The animals were rested for 1 week after arrival before use in an experiment. To exclude any animals exhibiting signs of corneal disease from the study, their eyes were examined for corneal clarity with a stereoscopic microscope at a magnification of × 40. The animals in this study were humanely treated in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

**Bacteria and preparation of inoculum.** *P. aeruginosa ATCC 19660* was obtained from the American Type Culture Collection (Rockville, Md.). The bacteria were grown to logarithmic phase in peptone-tryptic soy broth (PTSB) (0.25% tryptic soy broth [Sigma Chemical Co., St. Louis, Mo.]) and 5.0% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), and the culture was mixed with 15% (vol/vol) glycerol (Sigma), aliquoted, and frozen at −70°C. Stock cultures of *P. aeruginosa ATCC 19660* used to prepare the inoculum were maintained on PTSB slants (PTSB solidified with 1.7% agar [Difco]) at 4°C. Fresh slants were prepared every 2 weeks with frozen stocks as an inoculum.

To prepare a bacterial inoculum, a loopful of culture from a PTSB slant was used to inoculate 25 ml of PTSB. Cultures were grown at 37°C on a rotary shaker at 150 rpm for 18 h to an optical density at 540 nm of approximately 1.6. They were then centrifuged at 6,000 × *g* for 10 min at 15°C, washed once with sterile saline (0.85% NaCl [pH 7.2]), and resuspended in saline to a concentration of approximately 2.0 × 10^10 CFU/ml by using a standard curve relating viable counts to the optical density at 540 nm. *P. aeruginosa ATCC 19660* is proteolytic, hemolytic, and lipolytic, and under appropriate growth conditions, it produces exotoxins A, B, and C

Experimental mouse model of *P. aeruginosa keratitis*. A total of 24 young adult (6- to 8-week-old) and 24 aged (1.0- to 2-year-old) female Swiss mice were used

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* Corresponding author. Mailing address: Department of Anatomy/ Cell Biology, Wayne State University School of Medicine, 540 East Canfield Ave., Detroit, MI 48201. Phone: (313) 577-1061. Fax: (313) 577-3125. E-mail: lhazlett@med.wayne.edu.
in this study. The mice were anesthetized with isoflurane (AErrance, Anaquest, Madison, Wis.) and placed beneath a stereoscopic microscope at a magnification of x40. With a sterile 26-gauge needle, the corneal surface of the left eye of each animal was incised with three parallel 1.0-mm wounds. The incisions were centered over the pupillary axis and penetrated the epithelial cell basal lamina and into the superficial corneal stroma. A 5.0-μl bacterial suspending containing approximately 1.0 × 10^7 CFU was delivered topically onto the surface of the wounded cornea via a calibrated micropipette with a sterile disposable tip. At 6, 12, 24, and 48 h after the infection, six mice from each age group were sacrificed by cervical dislocation. The infected eyes were then carefully enucleated with scissors. The corneas were excised at the limbus with a sterile razor blade, and noncorneal tissue (i.e., sclera, iris/ciliary body) was removed by dissection. Corneal tissue was then processed for quantitation of cytokines, viable CFU, and PMN as described below.

Quantitation of corneal cytokines. Individual excised infected corneas from three aged mice and three young mice per time point were weighed and then homogenized in 1 ml of 50 mM phosphate-buffered saline (PBS; pH 7.2) plus 1% Tween 20 (Sigma) in sterile tissue grinders. Because eyes of aged mice are approximately twice the size of those of young mice, an average wet tissue weight for excised corneas obtained prior to homogenization so that cytokine concentrations are expressed as picograms of cytokine per milligram of corneal tissue. Uninfected corneas from age-matched naive mice were weighed, pooled (four corneas per 1 ml of PBS-Tween 20), and similarly homogenized as controls for cytokine ELISAs (40). Homogenates were centrifuged at 8,000 × g and 4°C for 20 min to pellet tissue fragments. Supernatants were aliquoted and immediately frozen at −70°C until assayed. IL-1β in the supernatants was quantitated with a kit from PerSeptive Diagnostics (Cambridge, Mass.) as specified by the manufacturer. IFN-γ and TNF-α were quantitated by capture ELISAs with monoclonal antibody pairs (PharMingen, San Diego, Calif.) as specified by the manufacturer. Recombinant murine IFN-γ (PharMingen) and TNF-α (Genzyme, Cambridge, Mass.) were used to generate standard curves for each assay. The sensitivities of the ELISAs for IL-1β, IFN-γ, and TNF-α are 0.125, 10, and 15 pg of per ml, respectively. Each cytokine assay was repeated at least twice. For statistical analyses, any samples that were below the range of sensitivity of the ELISA were considered negative for that cytokine.

Quantitation of viable bacteria and PMNs per cornea. Individual corneas were homogenized in 1 ml of sterile PBS plus 0.5% bovine serum albumin (Sigma Chemical) in sterile tissue grinders. To quantitate viable bacteria per cornea, a 0.1-ml aliquot of corneal homogenate was removed and serially diluted 1:10 in sterile PBS-bovine serum albumin aliquot (0.1 ml) of each dilution were plated in triplicate onto Pseudomonas isolation agar (Difco) plates. The plates were incubated for 24 h in a CO2 incubator with a water jacket (American Scientific Products, McGaw Park, Ill.) at 37°C under 5.0% CO2. The lower limit of detection of this technique is 100 CFU per cornea. For corneas with less than 100 CFU of viable bacteria, a value of 0 CFU/cornea was used to calculate the mean number of CFU. The values of CFU per cornea are expressed as log10. The smallest detectable amount of MPO activity in this assay is 100 CFU per cornea. For corneas with less than 100 CFU of viable bacteria, a value of 0 CFU/cornea was used to calculate the mean number of CFU. The values of CFU per cornea are expressed as log10. The number of viable bacteria in the corneas is expressed as the mean CFU of both eyes ± standard error of the mean (SEM).

Quantitation of PMNs in corneal tissue was performed by a MPO assay essentially as described by Kernacki and Berk (28). A 0.1-ml aliquot of PBS containing hexadecyltrimethylammonium bromide (CTAB; Sigma) was added to the corneal homogenates to give a final concentration of 0.5% CTAB and a total sample volume of 1 ml. Samples were freeze-thawed a maximum of three times and briefly sonicated once prior to centrifugation at 14,000 × g and 4°C for 20 min. An aliquot of the supernatant (0.1 ml) was mixed with 2.9 ml of 50 mM potassium phosphate buffer (pH 6.0) containing o-dianisidine dihydrochloride (16.7 mg/100 ml; Sigma) and hydrogen peroxide (0.005%). The change in absorbance at 460 nm was continuously monitored (with an LKB spectrophotometer) for 5 min. One unit of MPO activity is defined as the degradation of 1 mmol of peroxide/minute at 25°C, which gives a change in absorbance of 1.13 × 10^-1/2/min. One unit of MPO activity has been reported to be equivalent to about 2 × 10^5 PMNs (28). The smallest detectable amount of MPO activity in this assay is 0.01 unit, equivalent to approximately 10^3 PMNs (21). For corneas with less than 0.01 unit, a value of 0 PMNs/mg of cornea was used to calculate the mean number of PMN. Infiltrating PMNs in corneal tissue are expressed as the mean log number of PMNs ± SEM per milligram of cornea.

Gross ocular pathologic changes. The gross ocular pathologic changes observed in the young and aged Swiss mice were similar to those reported previously (22). At 6 and 12 h p.i., the corneas of mice of both ages were clear (grade 0). At 24 h p.i., a slight opacity covered the pupils (grade +1) in the young mice while a dense band of opacity (+1 to +2) near the corneoscleral limbus obscured part of the iris. In contrast, little or no corneal opacity (grade 0 to +1) was noted at 24 h p.i. in aged animals. Although the eyes of infected aged animals were clearly swollen, the corneas appeared to be as clear as those of an uninfected contralateral eye of an old or a young mouse. By 48 h p.i., enucleated infected eyes of aged animals were swollen to approximately twice the size of uninfected contralateral eyes or of infected eyes of young mice. A dense corneal opacity obscured the anterior chamber of the eyes of the young adults (grade +2 to +3). In contrast, at 48 h p.i., the anterior chamber in aged mice was not completely obscured by corneal opacity (grade +1 to +2), and a dense band of opacity, similar to that observed in young mice at 24 h after infection, was now seen at the limbus.

Viable bacteria per cornea and estimation of PMN. The mean log10 number of viable bacteria per cornea (± SEM) is shown in Fig. 1. Despite a trend of corneas from aged mice to contain more viable bacteria per cornea over time, there was no significant difference between the number of viable bacteria recovered from corneas of either aged or young mice at 6 and 12 h p.i. (P > 0.78). However, at 24 h p.i., 40 times more viable bacteria were recovered from corneas of aged mice than from those of young mice (P < 0.01). By 48 h p.i., corneas from both aged and young mice contained more than 7 log units of bacteria (P > 0.39).

The mean log10 number of PMN per milligram of cornea (± SEM) is shown in Fig. 2. At 6 h p.i., no MPO activity was found in homogenates of corneas from aged mice (< 3.0 log units of PMNs). In contrast, nearly 4.5 log units of PMNs/mg of tissue were found infiltrating the corneas of young mice (P < 0.008). At 12, 24, and 48 h p.i., there was no significant difference (P > 0.80) between aged and young mice in the number of infiltrating PMNs per milligram of cornea.
Corneal cytokine concentrations. TNF-α was not detectable (<15 pg) in any of the corneal homogenates, regardless of the age of the mouse, the time p.i., or the infection status. IL-1β and IFN-γ were not detected (<10 and 125 pg, respectively) in uninfected corneas of either young or aged mice.

The concentrations of IL-1β in the corneas of infected young and aged mice are shown in Fig. 3. At 6 h p.i., IL-1β was detected only in the corneas of young mice (P ≤ 0.04). At 12 h p.i., although the difference was not statistically significant (P ≥ 0.20), the corneas of young mice contained three times as much IL-1β than did those of aged mice. Paradoxically, this ratio reversed itself at 24 h p.i., with the corneas of aged mice containing three times as much IL-1β compared to its concentration in the corneas of young mice (P ≤ 0.002). IL-1β concentrations remained higher in the corneas of aged mice at 48 h p.i., but not significantly so (P ≥ 0.48).

The concentrations of IFN-γ in the corneas of infected young and aged mice are shown in Fig. 4. At 6 h p.i., there was no significant difference in IFN-γ concentrations between young and aged mice (P ≥ 0.81). However, at 12, 24, and 48 h p.i., the concentration of IFN-γ in the corneas of young mice was significantly higher compared to that in the corneas of aged mice (P ≤ 0.05).

DISCUSSION

The pathogenesis of P. aeruginosa keratitis probably results from a complex interaction between exoproducts secreted from the organism, such as elastase, alkaline protease, and exotoxin A (35–37), and by-products of the host inflammatory response, such as oxidative metabolites, collagenases, and other enzymes released from infiltrating PMNs (1). Endogenous host matrix metalloproteinases (MMPs) such as MMP2 and MMP9 (31, 32, 43) activated by the bacterial proteases may also contribute to the severe tissue damage characteristic of P. aeruginosa keratitis. Although PMNs may be responsible for much of this damage, they also play an essential role in the containment and resolution of the infection (2, 13, 14, 18, 21).

Our previous study (22) suggested that the up-regulation of ICAM-1 in young Swiss mice facilitated the recruitment of inflammatory cells to the infected cornea. In contrast, ICAM-1 expression in aged mice was not up-regulated above constitutively expressed levels, resulting in a delayed infiltration of inflammatory cells into the cornea. The results of that study led us to formulate the hypothesis that in this mouse model of P. aeruginosa keratitis, the more rapidly the inflammatory cells reach the bacteria, the faster the infection is controlled and the probability of restoring corneal clarity is enhanced. Previous studies performed by Berk et al. (2) and Hazlett et al. (19) with inbred strains of young mice supported this hypothesis; resistant strains of mice (i.e., those able to restore clarity after infection) such as DBA/2J initially have significantly more infiltrating leukocytes in the infected cornea than do susceptible strains such as C57BL/6J, and the resistant strains clear the bacteria within a shorter time. The theory that a prompt and efficient inflammatory response to P. aeruginosa infection equates...
to a resistant phenotype also finds support from a murine lung model of infection. Mice highly resistant to lung infection with \textit{P. aeruginosa} exhibited a more rapid recruitment of inflammatory cells (PMNs and macrophages) to the site of infection and a faster clearance of bacteria from the tissue than did a strain of mouse characterized as susceptible to infection (34).

In the present study, there were significantly fewer PMNs ($P \leq 0.008$), as determined by an MPO assay, in the corneas of aged mice at 6 h p.i. By 12 h p.i., although the difference was not statistically significant ($P \geq 0.20$), there were approximately three times the number of PMNs in the corneas of aged mice than in the corneas of infected young mice. These data confirm the histopathological observations of corneal wound sites and planimetric density determinations of inflammatory cells within a defined region of the anterior chamber (the visual axis) presented in our previous study (22). In the present study, there were eight times as many bacteria in the corneas of aged mice at 12 h p.i., although this difference was not statistically significant ($P \geq 0.37$). By 24 h p.i., there were 40 times as many bacteria in the corneas of aged mice as in the corneas of young mice. This difference was significantly different ($P \leq 0.01$). Taken together, these results support the hypothesis that the early delay in the migration of PMNs and other inflammatory cells in the ocular tissues of aged mice translated into a failure to control bacterial growth in the cornea.

Other studies from our laboratory and from the laboratories of other investigators suggest that the susceptibility of the aged outbred Swiss mice to \textit{P. aeruginosa} corneal infection may not be due entirely to the lack of ICAM-1 up-regulation and the resulting delay in infiltrating inflammatory cells. Several age-related changes in the phenotypes of the inflammatory cells themselves have been described. Hazlett et al. (15) examined the phagocytic capability of peripheral blood PMNs and macrophages recovered from \textit{P. aeruginosa}-infected corneas. PMNs and macrophages from aged animals exhibited an impaired ability to phagocytize particulate zymosan or \textit{P. aeruginosa} compared to similar inflammatory cells from young animals. Ding et al. (5) reported that caseinase-elicted peritoneal macrophages from aged BALB/c mice produced 50% less hydrogen peroxide in response to phorbol myristate acetate or opsonized zymosan and that the IFN-\gamma-induced release of hydrogen peroxide and nitric oxide was also 50% lower in aged than in young mice. Studies with peripheral blood macrophages and PMNs from elderly humans (60 to 100 years old [8]) and peritoneal macrophages from aged BALB/c mice and guinea pigs (10) demonstrated a significant decrease in the chemotactic response of these phagocytic cells with age. Collectively, all of these changes in the functionality of these phagocytic cells could contribute to the inability of the aged mouse to clear bacteria from the infected corneas.

The data from our previous study (22) also led us to hypothesize that a deficiency in proinflammatory cytokines such as IL-1\beta, IFN-\gamma, and TNF-\alpha in aged mice might contribute to their inability to up-regulate ICAM-1 expression. These cytokines enhance ICAM-1 expression 5- to 10-fold above constitutive levels on receptive cells in vitro (9). The literature is replete with studies presenting conflicting data on the effect of the age of an organism on cytokine production in both human and murine systems (20). For example, concentrations of cytokines are known to increase or decrease in mice as the animals age (12, 44), and which cytokines are affected appears to be dependent on the mouse strain. Other variables that may account for reported differences on how age may affect cytokine production include the immune cells studied (e.g., T lymphocytes versus macrophages), the origin of these cells (i.e., lymph node versus spleen, lung versus peritoneal cavity) and the culture conditions, the type of stimulation used (lipopolysaccharide versus a lectin), and the choice of cytokine assay used (ELISA versus bioassay). At present, this is the only known study to examine the concentrations of these cytokines in the corneas of either uninfected or \textit{P. aeruginosa}-infected young or aged mice.

In this study, TNF-\alpha, IL-1\beta, and IFN-\gamma were not detected in uninfected corneas of mice of either age. Lausch et al. (30) were also unable to find IL-1\beta in normal corneas excised from young BALB/c mice. IL-1\beta can be produced by a variety of cells, including monocytes, endothelium, fibroblasts, keratinocytes, and epithelium. However, Lausch et al. (30) were able to detect minimal amounts of TNF-\alpha (7 to 8 pg/cornea), but the sensitivity of their ELISA (7 pg/ml) was slightly higher than that of the assay used in our studies (15 pg/ml). TNF-\alpha is produced by PMNs, activated T lymphocytes, NK cells, and endothelial cells. Also, as expected, there was no expression of IFN-\gamma in uninfected corneas of either young or aged mice. IFN-\gamma is produced by activated T lymphocytes and NK cells. To date, there are no studies which show the presence of T lymphocytes or NK cells in the normal, uninfected corneas of mice.

After infection with \textit{P. aeruginosa}, TNF-\alpha was not detected in the corneas of either aged or young mice at any of the early times p.i. examined in this study. This finding agrees with data presented in a study by Kernacki and Berk (27), who reported detectable ocular concentrations of TNF-\alpha by ELISA at 6 days p.i. In our study, we did not test for corneal TNF-\alpha beyond 2 days p.i. One factor that could explain the lack of TNF-\alpha early in \textit{P. aeruginosa} infections of the cornea is exotoxin A. At nontoxic concentrations, this inhibitor of protein synthesis will decrease the production of TNF-\alpha (as well as that of IL-1\beta and IFN-\gamma) in vitro (41).

There are contradictory reports of whether IL-1\beta production by monocytes is increased (7, 39) or decreased (4, 26, 33) in the aged mice. There was more IL-1\beta at 6 and 12 h p.i. in the corneas of young outbred Swiss mice than in corneas of aged mice. Later in the infection, this trend had reversed itself, and there was more IL-1\beta in the corneas of aged mice at 24 and 48 h p.i. The initial increase in IL-1\beta concentration seen in the corneas of young mice could reflect the expression of this cytokine from noninflammatory cell sources such as the corneal endothelium and corneal fibroblasts. The increased IL-1\beta concentrations in the corneas of aged mice later in the infection could be the result of monocytes infiltrating the infected cornea. Future studies are needed to identify the source of IL-1\beta expression in the infected cornea and to determine if the cytokine produced in both the aged and young cornea is biologically active.

As with IL-1\beta, there are conflicting reports of whether IFN-\gamma production increases (6) or decreases (3) with age. In our study, there was significantly less IFN-\gamma in the corneas of aged mice than in the corneas of young mice 12 to 48 h p.i. Also, for both young and aged mice, there was a steady decline in IFN-\gamma concentrations in the corneas as the infection progressed. This may result from degradation of the cytokine by \textit{P. aeruginosa} alkaline protease and elastase (24, 38). Whether the disparate response of aged and young mice in IFN-\gamma production reflects a dysregulation of the early innate immune response of the aged mice, either by recruitment of fewer NK cells infiltrating the infected cornea or by a diminished capacity of these cells to secrete IFN-\gamma, is intriguing. These prospects have yet to be determined. For these studies, we have begun to examine the role of the innate immune response in young and aged mice by developing an inbred mouse model which paralleled...
els the disease response in the Swiss outbred model on both gross observational and histopathological levels.

In summary, the evidence provided by this study shows that the lack of ICAM-1 up-regulation in aged outbred mice after ocular challenge with *P. aeruginosa* correlates with a reduction in IL-1β and IFN-γ concentrations. This lack of ICAM-1 up-regulation evidently resulted in a delayed recruitment of a sufficient number of PMNs and other inflammatory cells into the infected corneas of aged mice to prevent an increase in the bacterial load. This increase in the number of bacteria probably contributes to the poor disease outcome observed in the aged mice.

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