Characterization of Arachidonic Acid Metabolism and the Polymorphonuclear Leukocyte Response in Mice Infected Intracorneally With Pseudomonas aeruginosa

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Purpose. To examine the activity of myeloperoxidase (MPO) and the concentrations of the proinflammatory metabolites of arachidonic acid (AA) in ocular tissue of mice that are either capable or incapable of restoring corneal clarity during an intraocular Pseudomonas aeruginosa infection.

Methods. For a period of 11 days after infection, whole eyes were enucleated and homogenized in buffer from mice given only an initial infection as well as from mice given a subsequent infection in the previously uninfected eye either 4 or 8 weeks after the initial infection. Tissue-free supernatants from the ocular homogenates were used for the determination of MPO activity by quantitating the conversion of specific substrate by spectrophotometric methods and for the quantitation of AA metabolites by ELISA.

Results. Overall, animals reinfected at 4 and 8 weeks had a lower inflammatory response when compared to the mice given only the initial infection. The lowest levels of LTB4 and MPO activity, indicators of PMN involvement, were observed in the the 8-week reinfected mice, which restored corneal clarity in an enhanced manner.

Conclusions. These results suggest that induced ocular PMN responses may play a role, in part, in the inflammatory response leading to the tissue destruction observed during ocular P. aeruginosa infection. Invest Ophthalmol Vis Sci. 1995;36:16-23.

Pseudomonas aeruginosa keratitis is one of the most destructive bacterial diseases of the cornea and is characterized by severe ulceration and extensive dissolution of the corneal stroma. Because of its rapid course and the frequent resistance of P. aeruginosa to most antibiotics, the infection is difficult to control and subsequently may result in visual impairment or even blindness. Many of the clinical features of P. aeruginosa keratitis have been reproduced in a variety of animal models. Previous studies from our laboratory have characterized C57BL/6 mice as susceptible to ocular P. aeruginosa infection because they were unable to restore corneal clarity after infection and subsequently progressed to corneal perforation, phthisis bulbi (shrinkage), or both. When C57BL/6 mice are administered a second infection in the previously uninfected contralateral control eye either 4 or 8 weeks after the initial infection, differing abilities to recover from the second infection were observed. When the subsequent infection was given 4 weeks after the initial infection, approximately 20% to 30% of the mice were able to restore corneal clarity within 4 weeks. However, when mice receiving the initial infection were given a subsequent reinfection 8 weeks after the initial infection, enhanced restoration of corneal clarity was observed. In these experiments, 89% of the animals restored corneal clarity within 3 to 6 days, whereas the remainder recovered within 14 days.

The host-derived inflammatory process has been implicated as an important contributor to the corneal disease seen during P. aeruginosa infection. One particular group of inflammatory mediators consists of oxidation products of arachidonic acid (AA) and includes products from both the cyclooxygenase and lipoxygenase pathways of AA metabolism. These mediators have been shown to originate from corneal epi-
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The role of PMNs in ocular tissue damage has been implicated in the pathogenesis of *P. aeruginosa* infections in both lung tissue and in burn wounds.14-19

The purpose of this study was to characterize the inflammatory response in mice that are either capable or incapable of restoring corneal clarity when intracornally challenged with *P. aeruginosa*. The parameters of inflammation to be characterized are myeloperoxidase activity and release of AA metabolites. Initially, C57BL/6 mice were administered an infection only in the left eye. Either 4 or 8 weeks after the initial infection, the mice were given a subsequent corneal infection in the previously uninfected contralateral control eye. A control group of age-matched, naive mice were simultaneously infected at the time of the reinfections. Ocular myeloperoxidase (MPO) activity, an enzyme found in high concentrations in the azurophilic granules of PMNs, was determined in the infected animals to give an estimation of infiltrating PMNs during infection. Concomitantly, concentrations of prostaglandin E2 (PGE2), leukotriene B4 (LTB4), and thromboxane B2 (TxB2) were measured in homogenates of infected ocular tissues at certain time points after infection to characterize the inflammatory mediator release during infection.

**MATERIALS AND METHODS**

**Bacteria**

Stock cultures of *P. aeruginosa* ATCC 19660 were stored at 4°C on tryptose agar slants (Difco Laboratories, Detroit, MI) and were used for the inoculation of 50 ml of broth medium containing 5% peptone (Difco) and 0.25% trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD). Strain 199660 is hemolytic, prototrophic, and lecithinolytic, and it produces exotoxin A. Cultures were grown on a rotary shaker at 37°C for 18 hours, centrifuged at 6,000g for 10 minutes at 4°C, washed three times with normal saline, and resuspended in 0.9% sterile nonpyrogenic saline (Travenol Laboratories, Deerfield, IL) to a concentration of 2 × 10^9 CFU/ml by using a standard curve relating viable counts to optical density at 440 nm.

**Infection of Animals**

Experiments in which mice were given an initial infection only were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals subsequently reinfected were treated as humanely as possible. Inbred C57BL/6j female mice (Jackson Laboratories, Bar Harbor, ME) (18 to 22 g) were used for these studies. All mice were age matched for the three different experimental groups, so that the animals receiving the initial infection only and the 4- and 8-week reinfections occurred at the same time. Before infection, the mice were lightly anesthetized with ether and placed beneath a stereoscopic microscope. The corneal surface was then incised with three 1-mm incisions using a sterile 26-gauge needle, taking care not to penetrate the anterior chamber or to damage the sclera. A bacterial suspension (5 μl) containing 1 × 10^8 CFU was topically delivered onto the surface of the wounded cornea by using a micropipette with a sterile, disposable tip. Mice were examined 24 hours later to ensure infection.

**Estimation of PMNs in Ocular Tissue**

Samples were assayed for MPO activity as previously described.27 Briefly, whole individual eyes were homogenized in 2 ml of 0.5% hexadecyltrimethylammonium bromide, (Sigma Chemical, St. Louis, MO) in potassium phosphate buffer (50 mM, pH 6.0). Whole eye homogenates, rather than just the corneas, were used in these studies for two reasons. First, it is difficult to obtain and standardize adequate corneal material from the mice, especially later in the infection because of the weakening of the tissue as a result of infection. Second, the corneal infections resulted in severe keratitis and extensive involvement of the sclera, with eventual development of phthisis; because of the involvement of ocular tissues in addition to the cornea, it was appropriate to use the whole eye rather than corneas alone. Four samples per time period (days 2, 5, 8, and 11 after infection) consisting of individual eyes were collected per experimental group of animals. These times were chosen because previous studies from our laboratory have shown that the C57BL/6 mice restore ocular sterility 9 to 12 days after infection and 7 to 9 days after either the 4- or 8-week reinfections.58,29 We have also determined that other inflammatory mediators (IL-1, IL-6, and TNF-α) approach baseline preinfection concentrations by this time in the infection.30 Samples were sonicated (Heat Systems Sonicator, Plainview NY) for 10 seconds in an ice bath immediately after collection. The samples were then freeze-thawed three times, after which they were sonicated one more time on ice for 10 seconds. The samples were centrifuged at 8000g for 20 minutes. An aliquot of the resulting supernatant (0.1 ml) was then mixed with 2.9 ml of potassium phosphate buffer (50 mM, pH 6.0) containing O-dianisidine dihydrochloride (16.7 mg/100 ml), (Sigma) and hydrogen peroxide (16.7 mg/100 ml), (Sigma).
Corneal Disaggregation for the Preparation of a Standard Curve Relating MPO Activity to Number of PMNs

The procedure for corneal disaggregation as described by Badenoch et al. was used in these studies with some minor modifications. Briefly, whole corneas from 10 animals infected 48 hours previously with P. aeruginosa strain 19660 were collected and used to prepare a population of PMNs for use in correlating units of MPO with numbers of infiltrating PMNs. The 10 infected corneas were placed in 10 ml of filter sterilized phosphate buffered saline (PBS) with 0.05% type IV collagenase (Sigma) and 0.25% type VI pancreatin (Sigma). The disaggregation procedure proceeded for 45 minutes at 37°C with stirring. After the incubation period, the enzymatic activity was inactivated by adding 2 ml of fetal calf serum. The sample was spun at 180g for 10 minutes and resuspended in 2 ml of PBS. Viable cell counts were determined by trypan blue exclusion staining, and the percentage of PMNs in the samples was determined by counting 400 nucleated cells stained by the Giemsa method. Serial two-fold dilutions of the original sample were used to produce a standard curve relating units of MPO to number of PMNs.

Quantitation of AA Metabolites During Ocular Infection

Samples for the AA metabolites, prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), leukotriene B\textsubscript{4} (LTB\textsubscript{4}), and thromboxane B\textsubscript{2} (Tx\textsubscript{B}\textsubscript{2}), were collected by sacrificing three animals per group per time period (preinfection and 1, 3, 5, 7, 9, and 11 days after infection) with individual eyes representing one sample. Eyes were enucleated after infection and homogenized in a sterile tissue grinder in 1.0 ml of iced ethanol after rinsing the entire eye in sterile saline to remove any contaminating blood obtained during the enucleation procedure. The net tissue weight of the eye was determined by subtracting the weight of the microcentrifuge tube containing 1.0 ml of ethanol from the weight of the same tube containing the entire enucleated eye. Samples were frozen at $-70^\circ$C until they were assayed. Upon removal from $-70^\circ$C, the samples were spun at 8,000g at 4°C for 20 minutes, and 800 \mu l of the resulting supernatant was transferred to another microcentrifuge tube for evaporation of the ethanol. The remaining pellets were resuspended in 1.6 \mu l of PBS with 3% BSA (Sigma). Samples were then assayed in a competitive inhibition ELISA (Advanced Magnetics, Cambridge, MA). The sensitivity for all three AA metabolites assays is 10 pg/ml. Results are reported as pg of AA metabolite per milligram of ocular tissue ± SD.

Statistical Analysis

The statistical differences at individual time points were determined by performing a one-way analysis of variance between the three experimental groups per time period. Significant differences were determined by using the F test at a confidence interval of 95%. Statistical analysis was done by using the Statview statistical package (Abacus Concepts, Berkeley, CA).
RESULTS

Determination of Numbers of PMNs Per Unit of MPO Activity

The calculations of numbers of PMNs per unit of MPO was determined by preparing standard curve of units of MPO activity versus numbers of PMNs by using PMNs collected from 48-hour infected eyes by corneal disaggregation. One unit of MPO activity has previously been described as that degrading 1 mmol of peroxide/minute at 25°C. The degradation of 1.0 mmol of peroxide has been reported to give a change in extinction of $1.13 \times 10^{-2}$/minute. In the studies herein, one unit of MPO activity was found to be equivalent to approximately $2 \times 10^5$ PMNs and is similar to that reported by both Williams et al. and Hobden et al. for rabbit PMNs.

Estimation of PMNs in Ocular Tissue During Infection

Comparison of the ocular activity of MPO during the initial and 8-week reinfections revealed significantly higher levels of enzyme activity in the eyes of animals receiving only the initial infection at all four time points examined (2, 5, 8, and 11 days after infection) (Fig. 1). An intermediate MPO response was observed in the mice given the 4-week reinfection, which was significantly different from both the initial infection and 8-week reinfection at days 5 and 8. Peak MPO activity for the mice receiving the initial infection only was found at day 5 after infection, whereas the groups receiving the 4- and 8-week reinfections were found to have peak activity at day 2, the earliest time point examined.

Determination of Ocular Response During Infection

Concomitant with the collection of samples for the analysis of AA metabolites during the initial and subsequent infections, grades for the ocular response were determined. Figure 2 shows the average ocular response for the three samples per experimental group obtained from mice at a given day after infection. As reported previously, most of the mice given an 8-week subsequent reinfection were able to restore corneal clarity within about 5 days after infection (grades 0 to 1). The animals given the initial and 4-week reinfections were able to restore corneal clarity within about 5 days after infection (grades 0 to 1). The animals given the initial and 4-week reinfections developed a similar (grade 3) severity of infection by day 3. The average severity of the ocular response in these mice was between 2.6 and 3 by days 9 to 11 after infection.

Ocular Concentrations of PGE2 During Initial and Subsequent Infections

Samples for the determination of all three metabolites were collected before infection and at 1, 3, 5, 7, 9, and 11 days after either an initial or a subsequent infection. As a separate control, uninfected mice that had only received the corneal abrasion necessary to initiate infection were examined for the concentrations of all three metabolites for the 11-day infection period. The only metabolite that showed any elevation as a result of wounding was PGE2 (data not shown). This elevation was not significant because wounding only produced levels of PGE2 equivalent to approximately 1/50 of that induced by the infection at the peak time of 5 days after infection. As another set of controls, AA metabolites were examined in the previously infected eyes of the 4- and 8-week reinfections and in the uninfected eyes of the initial infection concurrent with the active infections. Significant elevations above that of uninfected eyes from naive mice were not detected for any of the metabolites in these control groups (data not shown). The determinations for all AA metabolites during initial and subsequent infections were performed in duplicate experiments using different groups of animals for each experiment to ensure the kinetics and magnitude of the inflammatory response. The data for one of the experiments is shown in Figures 3, 4, and 5. When the concentrations of PGE2 were compared between the mice receiving the initial infection and the 8-week reinfection, significant differences were noted as early as 1 day and up to 9 days after infection (Fig. 3). Initially, the 8-week reinjected animals produced higher concentrations of PGE2, which peaked at 3 days after infection. After this, the mice receiving
FIGURE 3. Determination of ocular concentrations of PGE₂ produced during *P. aeruginosa* infection. Supernatants of ocular homogenates were assayed by ELISA for PGE₂. The data are presented as pg of PGE₂/mg of ocular tissue. Each sample represents an individual eye. Error bars indicate the standard deviation from the mean of three samples per experimental group per time point.

the initial infection only had a greater response through day 9. The peak time point for these animals was observed at day 5. Peak concentrations for the mice receiving the initial infection approached 375 pg/mg tissue. Peak concentrations for the 8-week reinfected animals approached 300 pg/mg tissue. The greatest response observed for the 4-week reinfected animals was approximately 150 pg/mg tissue at day 5 (Fig. 3). These animals consistently produced lower PGE₂ responses than the mice given initial and 8-week reinfections. The ocular concentrations were significantly different from the mice given the initial infection at days 5 through 11 and from those given the 8-week reinfection at days 1 through 11.

**Ocular Concentrations of LTB₄ During Initial and Subsequent Infections**

When the ocular concentrations of LTB₄ were compared between animals receiving the initial infection and the 8-week reinfection, a significantly greater response was found on days 1 through 9 in the mice receiving the initial infection (Fig. 4). By 9 to 11 days after infection, both groups of animals closely approached baseline preinfection concentrations because little LTB₄ was detected in either group at these times. Peak concentrations for both groups were observed at 3 days after infection, with the animals receiving the only initial infection producing more than 100 pg of LTB₄/mg of tissue, whereas the animals receiving the 8-week reinfection only produced peak concentrations of about 40 pg/mg of tissue. Comparable concentrations of LTB₄ were found in the mice given the initial and 4-week reinfection at all times tested (Fig. 4). Significant differences between the 4- and 8-week reinfected animals were evident at days 1 through 5 after infection. Peak concentrations of approximately 90 pg/mg of tissue were seen at day 3 in the 4-week reininfected animals. LTB₄ concentrations in these mice approached baseline values by 9 to 11 days after infection.

**Ocular Concentrations of TxB₂ During Initial and Subsequent Infections With *P. aeruginosa***

Comparison of the ocular concentrations of TxB₂ during the initial and 8-week reinfections revealed significantly higher concentrations of TxB₂ in the mice given the initial infection only at days 3 through 7 after infection (Fig. 5). The mice that received the 8-week reinfection had significantly higher TxB₂ concentrations at day 9, a time point where both groups were approaching resolution of the infection. Peak concentrations of approximately 150 pg/mg of tissue were observed at day 5 in the mice receiving only the initial infection. A peak of approximately 90 pg/mg of tissue was observed in the 8-week reinfected animals between days 3 and 5. Significant differences between the mice given the initial and the 4-week reinfections were found at days 3 through 7. The only difference between 4- and 8-week reinfected animals was observed at day 5 after infection. Peak concentrations of TxB₂ of approximately 75 pg/mg of tissue were observed in the 4-week reinfected animals at day 3 (Fig. 5); these concentrations approached baseline
cause platelet aggregation. Studies using spe-
dins and thromboxanes. These compounds are im-
portant in regulating vascular permeability and resis-
tance. The thromboxanes have also been shown to
be deleterious. Suppression of the cyclooxygenase
pathway, in this model, was shown to cause accelerated
ulceration whereas suppression of the lipoxygenase
pathway reduced the severity of the ulceration.

When the ocular concentrations of PGE$_2$, LTB$_4$, and TxB$_2$ were determined during the 11-day infec-
tion period, it was observed that the mice receiving
only the initial infection produced the greatest TxB$_2$
response when compared to the mice receiving the
4- and 8-week reinfections. There was essentially no
difference between the 4- and 8-week reinfected mice,
although the response in the 8-week reinfected mice
persisted somewhat longer than in the 4-week rein-
fected mice. Examination of PGE$_2$ concentrations
showed that the mice receiving the initial infection
produced the greatest PGE$_2$ response when compared
to both groups of mice receiving the subsequent rein-
fec tions. Overall, the 8-week reinfected animals pro-
duced a better PGE$_2$ response when compared with
the 4-week reinfected animals, whereas examination of
ocular LTB$_4$ concentrations showed essentially no
difference between the mice receiving the initial infec-
tion and the 4-week reinfec tion. The LTB$_4$
concentrations for the mice receiving the 8-week reinfec tion were significantly lower than for the mice receiving
the initial and the 4-week reinfections. Examination
of ocular MPO activity also supports the LTB$_4$
findings. MPO activity in the mice receiving the 8-week reinfection was significantly lower than that of mice receiving the initial and the 4-week reinfections. The mice rece-
viving the 4-week reinfection produced an intermedi-
ate response between the primary infection and the
8-week reinfected mice.

Decreased concentrations of all three inflamma-
tory mediators, as well as MPO activity, measured in
both the 4- and 8-week reinfected animals is a direct
correlation with the ocular bacterial load during infec-
tion because the peak bacterial load in mice receiving
only the initial infection has previously been reported
to approach $10^6$ CFU approximately 5 to 6 days after
infection. Sterility measured by quantitative plate
counts was observed in these mice by 9 to 12 days after
infection, whereas in 4- and 8-week reinfected animals,
peak numbers approaching $10^6$ CFU were observed at
day 3, and the infection cleared by 7 to 9 days. There
was no difference in the peak bacterial load or
the kinetics of clearance between the either the 4- or
8-week reinfected animals.

It is interesting, however, that there is a reversal
in the response of PGE$_2$ and LTB$_4$ induced between
the 4- and 8-week reinfected animals. In the 4-week

**DISCUSSION**

It has been suggested by various groups that host-
 inflammatory responses play an important role in the
corneal destruction seen during *P. aeruginosa* infec-
tion. Therefore, the purpose of the current
study was to characterize the inflammatory response
induced in mice that are either capable or incapable
of restoring corneal clarity after ocular infection with
*P. aeruginosa*. To achieve this, several parameters of
the inflammatory response induced by ocular infection
were quantitated and compared. These include
MPO activity as well as metabolites representing each
of the major oxidation pathways of AA, PGE$_2$, LTB$_4$, and TxB$_2$.

AA is a 20-carbon polyunsaturated fatty acid that
forms a portion of the phospholipid pool in cell mem-
branes. Release of AA from the cell membrane allows
this molecule to be metabolized into a variety of in-
flam matory compounds, such as leukotrienes, prosta-
glandins, and thromboxanes. Metabolism of AA by
the lipoxygenase pathway leads to the production of
leukotrienes and hydroxyeicosatetraenoic acids. The
leukotrienes, more specifically LTB$_4$, have been
shown to be important in chemotaxis and activation
of PMNs. Metabolism of AA by the cyclooxygenase
pathway leads to the production of both prostaglan-
dins and thromboxanes. These compounds are im-
portant in regulating vascular permeability and resis-
tance. The thromboxanes have also been shown to
cause platelet aggregation. Studies using spe-
cific inhibitors of either the lipoxygenase or cyclooxy-
genase pathways of AA metabolism in an ocular *P.
aeruginosa* model have suggested that the prostaglan-
din response may, in fact, be a protective response
whereas the PMN-inducing leukotriene response may
be deleterious. Suppression of the cyclooxygenase
pathway, in this model, was shown to cause accelerated
ulceration whereas suppression of the lipoxygenase
pathway reduced the severity of the ulceration.

AA Metabolites and PMN Response During *P. aeruginosa* Infection

**FIGURE 5.** Determination of ocular concentrations of TxB$_2$
produced during infection with *P. aeruginosa*. Samples were
collected and assayed as described in Figure 3.
reinfected animals, the LTB₄ concentrations are similar to that of the initial infection, but the PGE₂ response is significantly depressed. In the 8-week reinfected animals, the PGE₂ concentrations more closely parallel the initial infection, whereas there is a significant decrease in the LTB₄ response. Studies using specific inhibitors of both the cyclooxygenase and lipoxygenase pathways in ocular and nonocular inflammatory models have shown that use of a specific inhibitor will shift the metabolism of free AA to a supplemental pathway.²⁴⁻²⁶ These findings may explain in part this reversal between the 4- and 8-week subsequent reinfected groups because, on the basis of bacterial load, both reinfected groups have a similar intensity of an inflammatory stimulus, yet each individual infection preferentially metabolizes the free, nonmembrane-associated AA pool through different pathways.

In the model described herein, administration of a subsequent corneal Pseudomonas infection either 4 or 8 weeks after the initial infection results in the overall reduction of the inflammatory response as measured by MPO activity as well as by AA metabolites. The difference between administering the subsequent reinfection either 4 weeks or 8 weeks after the initial infection results in significantly lower levels of ocular LTB₄ and MPO activity in the 8-week reinfected mice, suggesting that a reduction in the infiltrating PMNs may in part contribute to the recovery of these mice.

Key Words

Pseudomonas aeruginosa, arachidonic acid, myeloperoxidase, polymorphonuclear leukocytes, keratitis

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References


