Mitigation of Neutrophil Infiltration in a Rat Model of Early *Staphylococcus aureus* Endophthalmitis

Michael J. Giese, 1,2 Sylvia A. Rayner, 1,2 Babak Fardin, 1,2 Humphrey L. Sumner, 1,2 Nora Rozengurt, 3 Bartly J. Mondino, 1,2 and Lynn K. Gordon 1,2,4

**Purpose.** Infectious endophthalmitis is characterized by neutrophil migration into the eye. The purpose of this study was to determine whether systemic neutrophil depletion mitigates the ocular influx of neutrophils during the early phases of experimental endophthalmitis.

**Methods.** Endophthalmitis was induced in rats by intravitreal injection of *Staphylococcus aureus*. Animals received a single systemic dose of an anti-neutrophil–depleting antibody (dAb) or normal rabbit serum (NRS) 6 or 12 hours after intravitreal injection. Inflammation was graded both in vivo and by histopathology. Myeloperoxidase (MPO) was used as a biomarker of neutrophil infiltration. Bacterial clearance was evaluated by determining the amount of viable bacteria recovered from ocular specimens.

**Results.** Rats that received dAb 6 hours after bacterial injection exhibited significantly lower clinical scores, MPO activity, fewer vitreous exudates, and higher vitreous bacterial counts at 24 hours (*P* < 0.05). As the neutrophil population returned in this group, measured by the number in the peripheral blood, increasing intraocular inflammation was observed. Rats receiving dAb 12 hours after vitreous injection also demonstrated significantly lower clinical scores, MPO activity and less vitreous exudates at the 24-hour time point (*P* < 0.05). No significant differences from the control were detected at any of the subsequent time points, except in bacterial counts and MPO activity.

**Conclusions.** Depletion of neutrophils early in the inflammatory response delayed the onset of severe ocular inflammation but also prevented adequate bacterial clearance. These results confirm the important role of neutrophils in ocular host defense during the early stages of experimental endophthalmitis.

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A major cause of postoperative endophthalmitis, *Staphylococcus aureus* was responsible for approximately 10% of infectious endophthalmitis cases in the Endophthalmitis Vitrectomy Study. Unfortunately, *S. aureus* endophthalmitis is associated with both rapid disease onset and poor visual outcome. Although the specific details of how *S. aureus* induces disease are not well characterized, it is known that *S. aureus* produces an estimated 34 products (e.g., toxins and enzymes) that have been shown to be important in several ocular diseases. Other *S. aureus* products such as cell wall components may also be responsible for induction of the ocular immunoinflammatory response. In addition, they may also play a role in direct microbe-induced damage in endophthalmitis. *S. aureus* products have also been shown to induce the expression of many host proinflammatory molecules. Injection of viable *S. aureus* into the vitreous chamber has been shown to induce the expression of intracellular adhesion molecule (ICAM)-1 and E-selectin. The exact contribution of host-derived inflammatory products to vision loss associated with infectious endophthalmitis is also poorly defined.

Bacterial clearance from the eye is dependent on a dynamic and complex host response characterized by the early recruitment and infiltration of neutrophils and subsequent microbial clearance. Neutrophils are the most common primary cell component of the innate immune response used in protection against extracellular bacterial infections. Early ocular infiltration of neutrophils is observed in experimental *S. aureus* endophthalmitis models. Bacterial clearance by neutrophils is accomplished by phagocytosis, generation of toxic reactive oxygen intermediates, and release of sequestered granule-associated enzymes, such as cathepsin G, myeloperoxidase (MPO), lactoferrin, and elastase. It is believed that release of these inflammatory mediators may result in enhanced local tissue damage. Thus, although neutrophils are an important component of microbial clearance in bacterial endophthalmitis, their products may also be responsible for some of the retinal toxicity in endophthalmitis and the subsequent loss of vision. The purpose of this study was to observe the effect of systemic neutrophil depletion in early experimental bacterial endophthalmitis on intraocular neutrophil infiltration and the clinical course of this disease.

**Materials and Methods**

**Bacteria and Animals**

In this study, a wild-type (WT) strain (RN6390) of *S. aureus* was used (provided by Ambrose Cheung, MD, Rockefeller University, New York, NY). RN 6590 is a laboratory strain that maintains its hemolytic pattern when propagated on sheep erythrocyte agar and produces α, β, and δ-hemolysin; lipases; and fibrinectin-binding protein. Isolates were stored in sheep blood at −70°C. In preparation for use, bacteria were plated onto sheep blood agar plates (BAPs) and incubated at 37°C. A discrete colony was subcultured into tryptic soy broth (Sigma Chemical Co., St. Louis, MO) and incubated overnight in a shaking water bath at 37°C. On the day of the experiment, the overnight bacterial culture was centrifuged at 1900g for 10 minutes, and the pellet was washed...
with lipopolysaccharide-free 0.9% sterile normal saline (NS), centrifuged, and resuspended in NS. The number of organisms in the suspension was estimated by taking the optical density (OD) at 600 nm (model DU-62; Beckman Coulter, Inc., Fullerton, CA), where one OD equals approximately 8 × 10^6 bacteria/mL. This suspension of S. aureus was adjusted by serial dilution with NS to yield a final concentration of approximately 120 bacterial L/ml and was used for intravitreal injection. This concentration was confirmed both before and after intravitreal injection by growth on BAPs.

Two hundred seventeen female Lewis rats (125–150 g) raised in a specific pathogen-free environment (Charles River Laboratories, Wilmington, MA) were used in this study. The number of animals varied in each group (dAb, n = 101; NRS, n = 67; untreated control, n = 49) and the number is listed next to the mean value for that group in Figures 2 and 3. Studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Endophthalmitis and Neutrophil Depletion**

After sedation with isoflurane and under direct visualization, paracentesis was performed on the dilated right eye. Each rat received a 10 µL intravitreal injection of S. aureus, according to a previously described procedure. Rats were killed at 24, 48, and 96 hours after injection, whereupon eyes were removed for MPO assays, bacterial quantitation, and histopathological evaluation. Rats received a single injection in the tail vein of a rabbit anti-rat neutrophil-depleting antibody (dAb; AIAAD51140; Accurate Chemical and Scientific Corp., Westbury, NY) that was diluted 1:5 with 0.9% injectable sodium chloride (Abbott Laboratories, North Chicago, IL). The volume injected was based on the weight of the rat at the time of the experiment (200 µL/200 g). Control rats received normal rabbit serum (NRS; CL1000-500S; Accurate Chemical and Scientific Corp.) at the same dilution or received no dAb or NRS (untreated). Preliminary animal studies showed that injection of the dAb produced a mean 67% decrease in total white blood cells (WBCs) at 24 hours. At 48 hours, a 52% decrease had occurred, and at 72 hours a 42% decrease was noted. Full recovery of peripheral WBC counts was observed after 96 hours in the uninfected animals. Injection of NRS had no effect on the WBC counts (data not shown). A differential blood count showed that this antibody primarily depleted the neutrophil population.

**Clinical Evaluation**

All animals were observed daily for signs of clinical inflammation with slit lamp biomicroscopy and direct ophthalmoscopy. Clinical inflammation was scored on a scale of 0 to 4 in four locations (conjunctiva, iris, anterior chamber, and vitreous). Formation of pupillary membranes and synechia were also scored and graded. The total clinical inflammatory score per eye was calculated by totaling each value in each category. The maximum score possible was 24. The total inflammation score in the dAb-treated group was analyzed from each eye, three through the optic nerve (level 2), and after intravitreal injection by growth on BAPs.

**Histopathology**

Rats were killed with CO₂ asphyxiation. For light microscopy, eyes were removed and immersion fixed in 4% formalin. After fixation, tissues were held in 50% alcohol until processed and embedded in paraffin, using standard techniques. The eye was oriented in the block so that an axis passing through the optic nerve and the center of the cornea and posterior pole was centralized. Nine µm sections were analyzed from each eye, three through the optic nerve (level 2), three above it (level 1), and three below it (level 3). All sections were stained with hematoxylin and eosin. All enucleated eyes underwent examination and determination of the inflammatory score in a masked fashion by one observer (NR). Neutrophils were counted in 12 specified high-power (×400) fields. Other special stains included Giemsa and Gram.

**Measurement of MPO Activity**

Eyes were removed at the designated times and placed in NS on ice. Each eye was then weighed and placed into a test tube containing 1 mL of potassium phosphate buffer (PPB; 50 mM [pH 6.0]). The eye was homogenized (model 2000; Omni International, Warrenton, VA) on ice and centrifuged at 292 g (Sorval RC-5B; DuPont, Kendro Laboratory Products, Asheville, NC) for 5 minutes at 4°C. Two hundred microliters of supernatant was removed and used for bacterial culture to quantify the number of S. aureus colony-forming units (CFU). The remaining material was sonicated (Microson ultrasonic cell disrupter; Heat Systems, Farmingdale, NY) on ice in PPB with 2 µL (10 µg of each) protease inhibitors (leupeptin [1.288x], antipain,107-109 and pepstatin A [P5318]; Sigma-Aldrich), phenylmethylsulfonyl fluoride (Sigma) and 0.5% hexadecyltrimethylammonium bromide (Sigma). In preliminary studies in which purified MPO was used, the protease inhibitors had no effect on the assay. Then the samples were freeze thawed three times and centrifuged at 8000g for 20 minutes. From the resultant supernatant, a 0.1 mL aliquot was mixed with 2.9 mL of PPB containing 0.0167% O-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide. MPO activity was measured by the change in extinction with time at 460 nm, as recorded by a spectrophotometer (model DU-62; Beckman). Units of MPO degrade 1 micromole of peroxide per minute at 25°C. The degradation of 1 micromole of peroxide produces a change in extinction per minute of 1.13 × 10⁻³, and therefore 1 U of MPO causes in the same change in extinction per minute.17

**Statistical Evaluation**

Mean clinical and histology scores and mean MPO levels and bacterial counts were compared with one-way analysis of variance (ANOVA). The significance of post hoc t comparisons was determined with the Fisher-Tukey least significant difference criterion. Data shown are presented as the mean ± SEM. Significance was determined at P < 0.05.

**RESULTS**

**Efficiency of Neutrophil Depletion**

Total WBC counts were performed at multiple time points after initiation of endophthalmitis. Experimental animals received systemic injections of dAb 6 or 12 hours after bacterial injection, and control animals received an injection of NRS at the same times or received neither (untreated). The induction of systemic injections of dAb 6 or 12 hours after bacterial injection, and control animals received an injection of NRS at the same times or received neither (untreated). The induction of endophthalmitis by intravitreal injection of S. aureus caused an initial increase in the total WBC counts in the infected untreated animals and in those control animals that received intravenous (IV) injections of NRS (Fig. 1). Injection of dAb 6 and 12 hours after S. aureus injection produced significant decreases in total WBC counts at the 24-hour time point, compared with the control. Peripheral neutrophil depletion was evident in the dAb-treated animals at all tested time points, although minimal recovery was observed starting at the 48-hour time point.

**Clinical Inflammation**

Rats treated with the dAb at 6 and 12 hours demonstrated lower clinical inflammatory scores at the 24-hour time point than control animals (P < 0.05; Figs. 2A, 3A). In the 6-hour dAb-treated group, the total inflammation score increased at the subsequent time points, whereas it decreased in both control groups. This change was significantly different at 48 hours between the dAb- and NRS-treated groups (P < 0.05) and from both control groups at 96 hours (P < 0.05). The clinical score in the 12-hour dAb group did not differ significantly from the control groups at both 48 and 96 hours.

All rats showed vitreous exudates developing behind the lens (Figs. 2B, 3B). Rats that received the dAb at either 6 or 12 hours showed anterior uveal tract inflammation, choroidal changes, and minimal retinal vessels. The retinal vessels were intact and the posterior retina was clear.
hours after injection demonstrated an interesting delay in onset of these exudates, corresponding to the depletion and reconstitution of the peripheral WBCs. At 96 hours, the exudate score in the 6-hour dAb group was significantly increased over the untreated and NRS-injected rats ($P < 0.05$). Although neutrophil depletion with dAb at 12 hours was associated with a significant lower vitreous exudate score at 24 hours ($P < 0.05$), no statistically significant differences were detected at the later time points.

**Bacterial Counts**

The number of viable *S. aureus* organisms in the eye was determined at 24, 48, and 96 hours after initiation of bacterial infection.
endophthalmitis. In untreated control animals, *S. aureus* was totally cleared by 96 hours (Figs. 2C, 3C). Neutrophil depletion early in the course of infectious endophthalmitis with dAb at 6 hours resulted in higher mean log bacterial counts at all time points (*P* < 0.05; Fig. 2C). In animals in which neutrophil depletion was delayed by an additional 6 hours (dAb treatment at 12 hours), higher bacterial counts were observed and showed significant differences compared with the untreated control group at 24 hours and the NRS control at 48 hours (Fig. 3C).

**MPO Activity**

MPO activity was used as a biochemical surrogate of neutrophil infiltration into the eye. In both neutrophil depleted groups, MPO activity was lower than the control at 24 and 48 hours (Figs. 2D, 3D). This result was consistent with histopathologic data and the decreased vitreous exudate scores. At 96 hours, the MPO activity in the early neutrophil depletion group, dAb at 6 hours, was statistically higher than the activity detected in the control (*P* < 0.05). This observation is consistent with the declining bacterial counts and worsening clinical and histopathologic scores in the same group of animals (Figs. 2D, 3D). However, in the 12-hour treated group, MPO activity returned to the control levels.

**Histopathology**

Representative appearances of anterior segment inflammation at 24 and 48 hours after initiation of bacterial endophthalmitis are depicted in Figure 4. Photomicrographs in Figure 4A represent control animals who received IV NRS. Significant inflammation was apparent at 24 hours and increased at 48 hours after onset of endophthalmitis. Figure 4B documents the decreased intraocular inflammation observed in animals systemically depleted of neutrophils through the use of intravenous dAb at 6 hours after bacterial exposure. The mean histologic inflammatory score in bacterial endophthalmitis with neutrophil depletion in the early stages of infection was significantly reduced (*P* < 0.05) at 24 hours in comparison to the saline and untreated control groups (Fig. 4).

**FIGURE 3.** Effect of neutrophil depletion 12 hours after *Staphylococcus aureus* injection. (A) Clinical inflammation. (B) Vitreous exudate. (C) Vitreous bacterial counts. + *P* < 0.05 between dAb and NRS, # *P* < 0.05 between dAb and untreated. (D) MPO activity. + *P* < 0.05 between dAb and untreated. *P* < 0.05 between dAb and control groups in all panels. The number of animals in each group is listed next to the mean.

**FIGURE 4.** Histology of early bacterial endophthalmitis. Photomicrographs of the anterior segments of representative rat eyes after initiation of bacterial endophthalmitis are shown. The eyes were enucleated 24 and 48 hours after onset of bacterial endophthalmitis, formalin-fixed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Control animals received an intravenous injection of NRS, without systemic depletion of neutrophils (A). Experimental animals received an intravenous injection of a neutrophil-depleting antibody (dAb) 6 hours after intraocular bacterial inoculation (B). Bar, 50 μm.
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Either 6 or 12 hours after initiation of endophthalmitis was not statistically significant. The inflammatory scores for animals that received IgG at either 6 or 12 hours after initiation of endophthalmitis were not statistically significant (data not shown). Data are demonstrated for both 24 and 48 hours after onset of endophthalmitis.

**Discussion**

Visual recovery after bacterial endophthalmitis is varied and depends on many factors. These include the time between onset and antimicrobial therapy, expression of bacterial virulence factors, and activity of the host immune response. Although the host immune response is required for bacterial clearance, release of potent intracellular mediators from activated neutrophils may produce local tissue damage, which may cause permanent loss of function of the neurosensory retina. Therefore, an optimal treatment approach may include recruitment of enough activated inflammatory cells that would allow complete bacterial clearance while minimizing secondary host-related toxicity. One way to achieve this is through immunosuppression with intraocular steroids as an adjunctive therapy. This is a controversial clinical practice, with both beneficial and detrimental effects reported. This nonspecific immunosuppression could be replaced by targeted immunotherapeutic modulations that permit fine-tuning of the intraocular inflammatory response. In this study, we addressed the selective reduction of inflammatory neutrophils in the course of bacterial endophthalmitis.

Our previous work in a rat endophthalmitis model showed that clinically observed intraocular infection was associated with upregulation of inflammatory chemoattractants and an influx of inflammatory cells 6 hours after inoculation with bacteria. In this current work, we selectively depleted systemic neutrophils at time points known to be associated with both clinical infection and intraocular influx of inflammatory cells. Systemic neutrophil depletion, to approximately 10% of normal, was observed within 24 hours after intravenous administration of an anti-neutrophil antibody. Systemic neutrophil depletion to this level was initially accompanied by a decreased intraocular neutrophil infiltrate, as judged both by clinical scores, histologic examination, and MPO assay. This indicated that, even in the presence of early, active infection and upregulated intraocular inflammatory cytokines and chemokines, modulation of the intraocular inflammatory response is possible.

When delivered 6 hours after intraocular bacterial inoculation, neutrophil-depleting antibody yielded a number of significant results. First, the intraocular inflammation was initially reduced as evidenced at the 24-hour time point by reduced clinical inflammatory scores, vitreous exudate scores, and MPO activity. However, the vitreous bacterial counts were significantly elevated at the 24-hour time point, indicating that, in the absence of exogenous pharmacologic antibiotic agents, the remaining intraocular neutrophils were incapable of adequately clearing the bacterial load. This inability to clear bacteria adequately could be a function of the reduced number of neutrophils; or, alternatively, the presence of the anti-neutrophil antibody may have impaired the antimicrobial function of the remaining neutrophils. Additional studies would be required to differentiate between these two possibilities.

Recovery of the peripheral neutrophil population to the pretreatment levels began at approximately the 48-hour time point and was complete within 96 hours. In the presence of ongoing bacterial infection, this recovery was associated with a vigorous accumulation of intraocular neutrophils. It is important to note that no specific antibacterial therapy was used in this study. It is hypothesized that the concomitant use of intraocular bacterial or bacteriostatic therapy at the time of clinically evident infection would have abrogated the rebound inflammation. Additional studies will be designed to test this prediction.

Neutrophil depletion achieved using anti-neutrophil antibody 12 hours after intraocular injection of bacteria was associated with a different clinical course. Bacterial clearance was not significantly different between the neutrophil-depleted animals (dAb12) and the control infected animals. This is evidence that, in the absence of specific antimicrobial agents, delaying the onset of neutrophil depletion for an additional 6 hours allowed adequate bacterial clearance. Decreased intraocular neutrophil-associated inflammation was also observed at the 24-hour time point after neutrophil depletion at 12 hours, as judged by intraocular MPO activity.

Depletion of peripheral neutrophils is a strategy that has been used experimentally in rat models of microvascular injury in experimental pancreatitis, lung, and hepatic injury during sepsis, and intestinal infections induced by severe burn injuries. Previous investigations also suggest a role for activated PMNs in bacterial clearance and production of corneal damage during bacterial keratitis. In these studies, neutrophil depletion and the resultant neutropenia was performed before initiation of keratitis through the use of total body irradiation. Neutropenia in these studies was associated with decreased neutrophil infiltration, less corneal edema, and reduced bacterial clearance in the setting of a Pseudomonas aeruginosa corneal infection. These animal models all demonstrate that systemic neutrophil depletion results in a transient reduction in tissue inflammatory infiltrate and decreased local tissue damage. These studies point to the important role of neutrophils and their inflammatory mediators as being responsible for the tissue damage observed in the setting of severe infection.

In the present study, we confirm that intraocular recruitment of inflammatory cells, which may play a role in retinal toxicity and loss of function, could be modulated during active infectious endophthalmitis. This abrogation is achieved despite intraocular expression of inflammatory chemokines during early bacterial infection. This study used anti-neutrophil antibodies to produce a transient neutrophil depletion and de-
crease in intraocular inflammation early in the course of infectious endophthalmitis. Continued neutrophil depletion using a second anti-neutrophil treatment was not performed but it is anticipated that this will prolong the suppression of intraocular inflammation. Future studies will address the final outcome of infectious endophthalmitis after neutrophil depletion and will investigate the ability of antimicrobial agents to promote bacterial clearance after neutrophil depletion. Additional work is needed to dissect the pathophysiology of loss of retinal function during infectious endophthalmitis—particularly, to investigate the contribution of bacterial products, live bacteria, and the host response in producing permanent damage. These studies are necessary to determine the best tools for modulating the host response during active intraocular infection to reduce permanent loss of vision in infectious endophthalmitis.

References