

# Phospholipase A<sub>2</sub> Activity in Normal and *Staphylococcus aureus*-Infected Rabbit Eyes

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**PURPOSE.** To quantify phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity in normal rabbit eyes and in eyes with *Staphylococcus aureus* keratitis.

**METHODS.** PLA<sub>2</sub> was assayed by the killing of *S. aureus* at 33°C or by the release of arachidonic acid from *S. aureus* labeled with radioactive oleic acid. Rabbit corneas were intrastromally injected with 100 log phase colony-forming units (CFU) of *S. aureus* 8325-4. The activity of myeloperoxidase (MPO) and PLA<sub>2</sub> were quantified in ocular tissues.

**RESULTS.** The PLA<sub>2</sub>-mediated killing of *S. aureus* by normal rabbit tears decreased by more than 70% as the rabbits aged from 10 to 28 weeks and by nearly 50% from early morning to afternoon. In rabbits with *S. aureus* keratitis, the activity of PLA<sub>2</sub> and MPO increased proportionally with time from 5 to 25 hours postinfection (PI), as measured in ocular tissues. PLA<sub>2</sub> activity increased fivefold in tears from infected eyes collected at 25 hours PI compared with normal tears ( $P \leq 0.0001$ ), whereas a ninefold increase was found in aqueous humor of infected eyes at 25 hours PI ( $P \leq 0.0001$ ). Infected eyes demonstrated a significant increase in MPO activity compared with uninfected eyes beginning at 10 hours PI for the aqueous humor ( $P = 0.03$ ), at 16 hours PI for the tear film ( $P = 0.0024$ ) and at 22 hours PI for the corneal homogenate ( $P = 0.0007$ ).

**CONCLUSIONS.** The decrease in PLA<sub>2</sub> activity in the rabbit eye with age or after sleep and its increase during sleep or with the progression of infection are consistent with its role as an innate host defense factor. (*Invest Ophthalmol Vis Sci.* 2003;44:197-202) DOI:10.1167/iops.02-0548

The ocular tear film contains numerous components that serve as host defense molecules. This innate defense against bacteria has traditionally been attributed to complement, lysozyme, defensins, lactoferrin, and IgA.<sup>1-7</sup> However, group II phospholipase A<sub>2</sub> (PLA<sub>2</sub>) is increasingly being recognized for its antibacterial activity against Gram-positive bacteria<sup>8-12</sup> and its important role as a defense molecule in the eye.<sup>13-15</sup>

PLA<sub>2</sub> is secreted from a number of cells,<sup>16</sup> including polymorphonuclear leukocytes (PMNs), and is found at high concentrations in inflammatory fluids<sup>17</sup> and human tears.<sup>13,18</sup> Group II PLA<sub>2</sub> is present in platelets and numerous fluids and tissues, including Paneth cells, synovial fluid, seminal plasma,

and prostatic gland cells.<sup>18,19</sup> The mammalian secretory group II PLA<sub>2</sub> (14 kDa) is mobilized at sites of inflammation and exerts potent antibacterial activity against Gram-positive bacteria.<sup>9</sup> PLA<sub>2</sub> is also known to play a role in the initiation and propagation of inflammatory processes.<sup>20</sup>

Numerous studies have been conducted in an attempt to elucidate the role of PLA<sub>2</sub> as a bactericidal host defense molecule. Weinrauch et al.<sup>11</sup> and Dominiacki and Weiss<sup>9</sup> have reported that PLA<sub>2</sub> from leukocytes can rapidly kill *Staphylococcus aureus*. Nevalainen et al.<sup>21</sup> have demonstrated a high concentration of group II PLA<sub>2</sub> in tears (1.451 mg/L) of healthy individuals. In addition, the antibacterial properties of PLA<sub>2</sub>, particularly against *S. aureus*, have been demonstrated in rabbit tears and human tears by Moreau et al.<sup>14</sup> and Qu and Lehrer,<sup>13</sup> respectively.

*S. aureus* is frequently associated with bacterial conjunctivitis and keratitis.<sup>22</sup> *S. aureus* is the most common agent of bacterial keratitis in many human populations,<sup>23-26</sup> both in normal corneas and previously compromised corneas.<sup>22,24</sup> *S. aureus* corneal infections occur primarily in contact lens wearers and patients with defects in their ocular immunity.<sup>22,27,28</sup> Moreover, bacterial keratitis mediated by *S. aureus* is a serious condition that can result in loss of vision.<sup>29,30</sup>

Although *S. aureus* is a common corneal pathogen, efforts to achieve a corneal infection by application of these bacteria to scarified corneas have been unsuccessful. Whereas the topical application of *Pseudomonas aeruginosa* to a scarified rabbit cornea results in keratitis,<sup>31</sup> *S. aureus* does not elicit keratitis in scarified rabbit eyes.<sup>32</sup> Topical inoculation of large numbers of *S. aureus* on the scarified rabbit cornea can result in inflammation, but an actual infection with bacterial replication characteristic of keratitis is not produced. Moreau et al.<sup>14</sup> have shown that *S. aureus* is rapidly killed in the tear film and in vitro when incubated with rabbit tears, except when inhibitors of PLA<sub>2</sub> are present. Positively charged molecules, such as spermidine or certain topical anesthetics, can inhibit this bactericidal reaction of PLA<sub>2</sub> by binding to the bacterial surface.<sup>14</sup> Spermidine has been found to protect bacteria in the tear film, allowing them to infect the scarified rabbit cornea.<sup>15</sup>

Because PLA<sub>2</sub> functions in such an important manner in protecting the cornea, further analyses of these reactions were undertaken in this study. The results demonstrate that the activity of PLA<sub>2</sub> in normal rabbit tears decreased with age and after periods of sleep, yet increased multifold in ocular tissues on infection with *S. aureus*.

## METHODS

### Bacterial Strains and Growth

*S. aureus* strain 8325-4 was used in this study and has previously been used in the rabbit intrastromal injection model of keratitis.<sup>33-35</sup> Bacteria were grown overnight in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) at 37°C and then subcultured to log phase (OD<sub>650</sub> = 0.3) under the same conditions.

### Rabbits

New Zealand White rabbits used in these studies were maintained in accordance with the ARVO Statement for the Use of Animals in Oph-

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thalmic and Vision Research. Rabbits were housed on a 12-hour light-dark cycle, changing to darkness at 6 PM and to light at 6 AM.

### Collection of Rabbit Tears

Microliter capillary pipettes were placed into the cul de sac of rabbit eyes and allowed to fill by capillary action. Volumes of 5 to 10  $\mu\text{L}$  were collected from each eye at any one time point. Unless otherwise stated, tears were collected in the midmorning. Tears were not collected from any rabbit on two consecutive days.

To determine the effects of age on PLA<sub>2</sub> activity, tears were collected from both eyes of 10-week-old rabbits and subsequently collected every 4 weeks thereafter, for a total of 32 weeks. Tears collected from rabbits were pooled at each monthly interval and stored at  $-70^{\circ}\text{C}$  until assayed.

To determine the effects of collection time on PLA<sub>2</sub> activity, tears were collected at one time point per day, with 2 days between before collecting tears from rabbits for the next time point. Tears were collected individually from both eyes of each rabbit and stored at  $-70^{\circ}\text{C}$  until assayed.

### Aqueous Humor Collection

A 26-gauge needle on a 1-mL syringe was inserted through the cornea into the anterior chamber of anesthetized rabbits and 0.1 mL of aqueous humor was removed.

### Keratitis Model

*S. aureus* (100 CFU in 10  $\mu\text{L}$ ) was intrastromally injected into both eyes of rabbits that had been systemically anesthetized with ketamine and xylazine and topically anesthetized with proparacaine, as previously described.<sup>33-35</sup> The inocula were plated onto tryptic soy agar (TSA; Difco) plates to confirm the number of colony-forming units injected. At various times postinfection (PI; 5, 10, 16, 22, and 25 hours), rabbits were killed by intravenous injection with a pentobarbital sodium solution (100 mg/mL; The Butler Co., Columbus, OH). Corneas were aseptically removed and homogenized as previously described.<sup>33-35</sup> Briefly, corneal homogenates were serially diluted in sterile phosphate-buffered solution (PBS), aliquots (0.1 mL) were inoculated onto TSA plates and the plates incubated at  $37^{\circ}\text{C}$  for 24 hours. Colonies were counted, and colony-forming units per cornea were expressed as log values.

### Myeloperoxidase Activity Assay

The amount of myeloperoxidase (MPO) activity was quantified in samples of ocular swabs, aqueous humor, and corneal homogenates, as described previously.<sup>36-38</sup> MPO activity in the tear film was assayed by swabbing each eye with a Dacron swab and subsequently soaking each swab in a sterile solution of 0.5% hexadecyltrimethylammonium bromide (CTAB; Sigma Chemical Co., St. Louis, MO), as previously described.<sup>36-38</sup> The MPO microtiter assay used an *o*-dianisidine-based colorimetric reaction. Reactions were incubated at room temperature and the change in optical density at 450 nm was determined every 2 minutes for 12 minutes. The units of MPO were calculated as previously described for the microtiter plate assay.<sup>36-38</sup> One unit of MPO activity was equivalent to approximately 100,000 PMNs. All assays were performed in triplicate.

### Protein Determinations

The bicinchoninic acid (BCA; Sigma) assay for microwell plates was used for the quantification of total protein in tears and aqueous humor. Briefly, dilutions of samples of aqueous humor or tears (in triplicate) were added to wells containing BCA working buffer (Sigma). A set of protein standards of bovine serum albumin (BSA; Sigma) was prepared and added to wells containing buffer. After incubation at  $37^{\circ}\text{C}$  for 1 hour, the plate was cooled to room temperature before the absorbance was measured at 560 nm on a microtiter plate reader. A standard curve was prepared by plotting the average blank-corrected absorbance reading for each BSA standard versus its concentration in micrograms

per milliliter. Using the standard curve, the protein concentration of aqueous humor or tear samples was determined.

### Radioactive Labeling of *S. aureus*

*S. aureus* strain 8325-4 was radioactively labeled as previously described.<sup>14</sup> Briefly, bacteria were grown overnight in M9 synthetic medium with amino acids, subcultured to log phase with 0.5  $\mu\text{Ci}/\text{mL}$  of <sup>14</sup>C-labeled oleic acid, and then incubated at  $37^{\circ}\text{C}$  for 2.5 hours. After incubation, the bacteria were centrifuged, the supernatant removed, the bacterial pellet resuspended in TSB, and incubated for 30 minutes at  $37^{\circ}\text{C}$ . The bacteria were then washed in PBS with 1% BSA, resuspended in PBS, and stored at  $-70^{\circ}\text{C}$ .

### Determination of Phospholipase A<sub>2</sub> Activity

PLA<sub>2</sub> activity in tears and aqueous humor was determined using radioactively labeled *S. aureus*, as previously described.<sup>14</sup> Briefly, radiolabeled bacteria were incubated with either tears or aqueous humor (or 1:5 dilution thereof in Tris-HCl buffer, pH 7.5), with 2 mM CaCl<sub>2</sub> added. The mixtures were incubated for 30 minutes at  $33^{\circ}\text{C}$ . Reactions were terminated with the addition of ice-cold BSA, and samples were centrifuged to pellet the bacteria. An aliquot of supernatant was used to quantify, by liquid scintillation counting, the products of hydrolysis as counts per minute (cpm). All assays were performed in triplicate.

The PLA<sub>2</sub> content of rabbit tears was also determined with a bactericidal assay in which log phase 8325-4 grown in a modified M9 synthetic medium was used, as previously described.<sup>14</sup> Bacteria (100–200 CFU in 15  $\mu\text{L}$ ) were mixed with rabbit tears (1–40  $\mu\text{L}$ ), and the mixtures were incubated at  $33^{\circ}\text{C}$  for 4 hours. After incubation, aliquots were cultured on TSA in triplicate. Controls included bacteria in M9 medium, PBS, or heat-inactivated tears (heated at  $80^{\circ}\text{C}$  for 5 minutes). The number of colony-forming units was determined from the colony count after 24 hours of incubation. All assays were performed in triplicate.

### Statistical Analysis

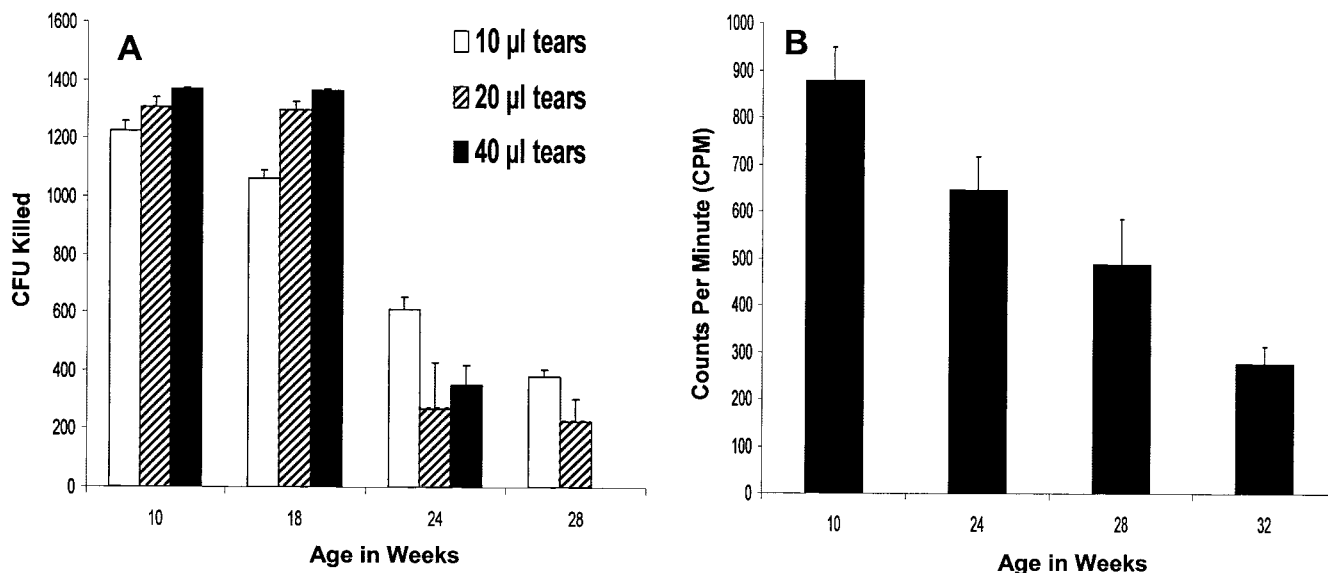
The mean and the SEM for PLA<sub>2</sub> assays (colony-forming units per bactericidal assay reaction or counts per minute per assay reaction), colony-forming units per cornea, units of MPO, and protein concentration were determined by computer (SAS software; SAS, Cary, NC). Statistical analyses were performed using a one-way nested analysis of variance on each group. Protected *t*-tests were then determined between least-square means derived from each variance analysis on each group.  $P \leq 0.05$  was considered significant.

## RESULTS

### Effect of Age on PLA<sub>2</sub> Activity of Rabbit Tears

To analyze the role of PLA<sub>2</sub> in the bactericidal action of normal rabbit tears, tears were collected from rabbits ( $n = 4$ ) at regular monthly intervals, and the survival of *S. aureus* mixed with these tears was determined. The bactericidal activity of the tears remained essentially equivalent from 10 to 18 weeks, declined significantly at week 24 ( $P \leq 0.0001$ ), and was undetectable in tears collected at week 28 (40  $\mu\text{L}$  of tears; Fig. 1A).

PLA<sub>2</sub> activity in these tears was also determined by measuring the release of arachidonic acid from radiolabeled *S. aureus*. Pooled tears collected from young rabbits ( $n = 4$ , 10 weeks old) demonstrated relatively large amounts of PLA<sub>2</sub> activity, as demonstrated by the counts per minute liberated. However, a significant decline in the amount of PLA<sub>2</sub> activity in tears was observed by 28 weeks (Fig. 1B,  $P = 0.0005$ ), compared with those tears collected at 10 weeks of age and declined even further by 32 weeks of age (Fig. 1B,  $P \leq 0.0001$ ).



**FIGURE 1.** Effect of age on bactericidal and enzymatic PLA<sub>2</sub> activity in rabbit tears. (A) Tears (10, 20, or 40 µL) collected from rabbits ( $n = 4$ ) at monthly intervals were incubated with log phase *Staphylococcus* for 4 hours, and the mixture was cultured to determine remaining colony-forming units. Controls for bacterial viability included bacteria incubated in M9 medium or PBS. The number of colony-forming units of bacteria remaining after incubation with inactivated tears (heated at 80°C for 5 minutes) was used to determine the maximum colony-forming units achievable without killing per volume of tears. Data are expressed as the mean  $\pm$  SEM. (B) *Staphylococcus* labeled with <sup>14</sup>C-oleic acid was incubated with rabbit tears at 33°C for 30 minutes. Reactions were terminated with the addition of BSA and then centrifuged to pellet bacteria. An aliquot of each supernatant was removed and radioactivity quantified by liquid scintillation counting as counts per minute. Data are expressed as the mean  $\pm$  SEM.

### Effect of Time of Day on PLA<sub>2</sub> Activity in Rabbit Tears

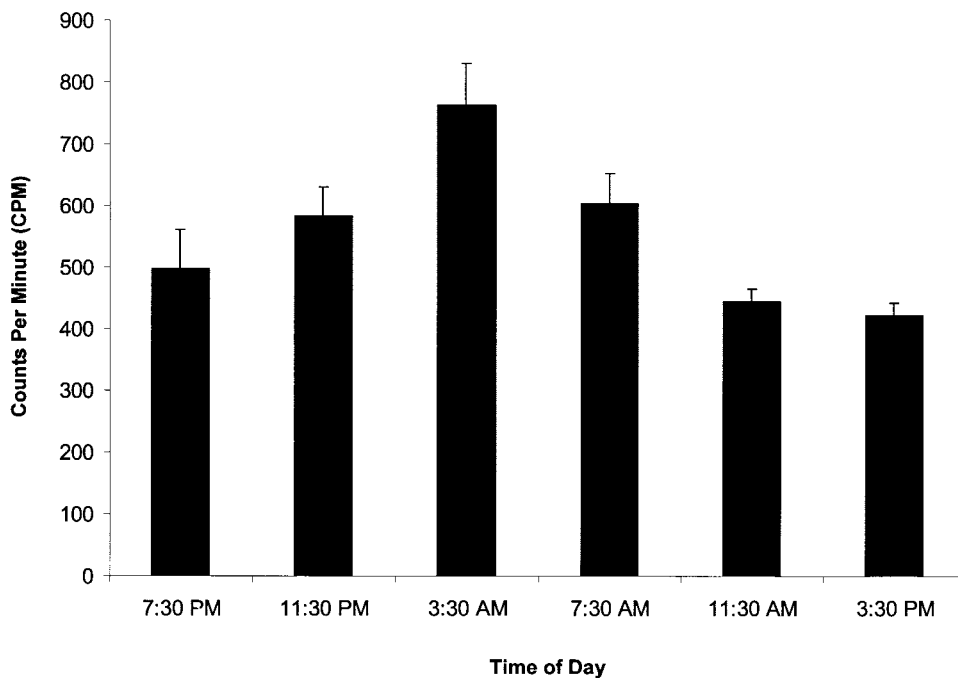
To further explore aspects of PLA<sub>2</sub> activity, tears were collected from rabbits ( $n = 6$ ) at six times during the day and night (3:30 AM, 7:30 AM, 11:30 AM, 3:30 PM, 7:30 PM, and 11:30 PM). The activity of rabbit tears in release of labeled arachidonic acid from bacterial membranes was tested using <sup>14</sup>C-labeled *S. aureus*, as previously described. Those tears collected early in the morning (3:30 AM) demonstrated significantly more release of radiolabeled arachidonic acid from bacterial membranes than did tears collected at 7:30 AM ( $P =$

0.01), 11:30 AM ( $P = 0.01$ ), 3:30 PM ( $P = 0.0075$ ), 7:30 PM ( $P = 0.0003$ ), and 11:30 PM ( $P = 0.01$ ; Fig. 2).

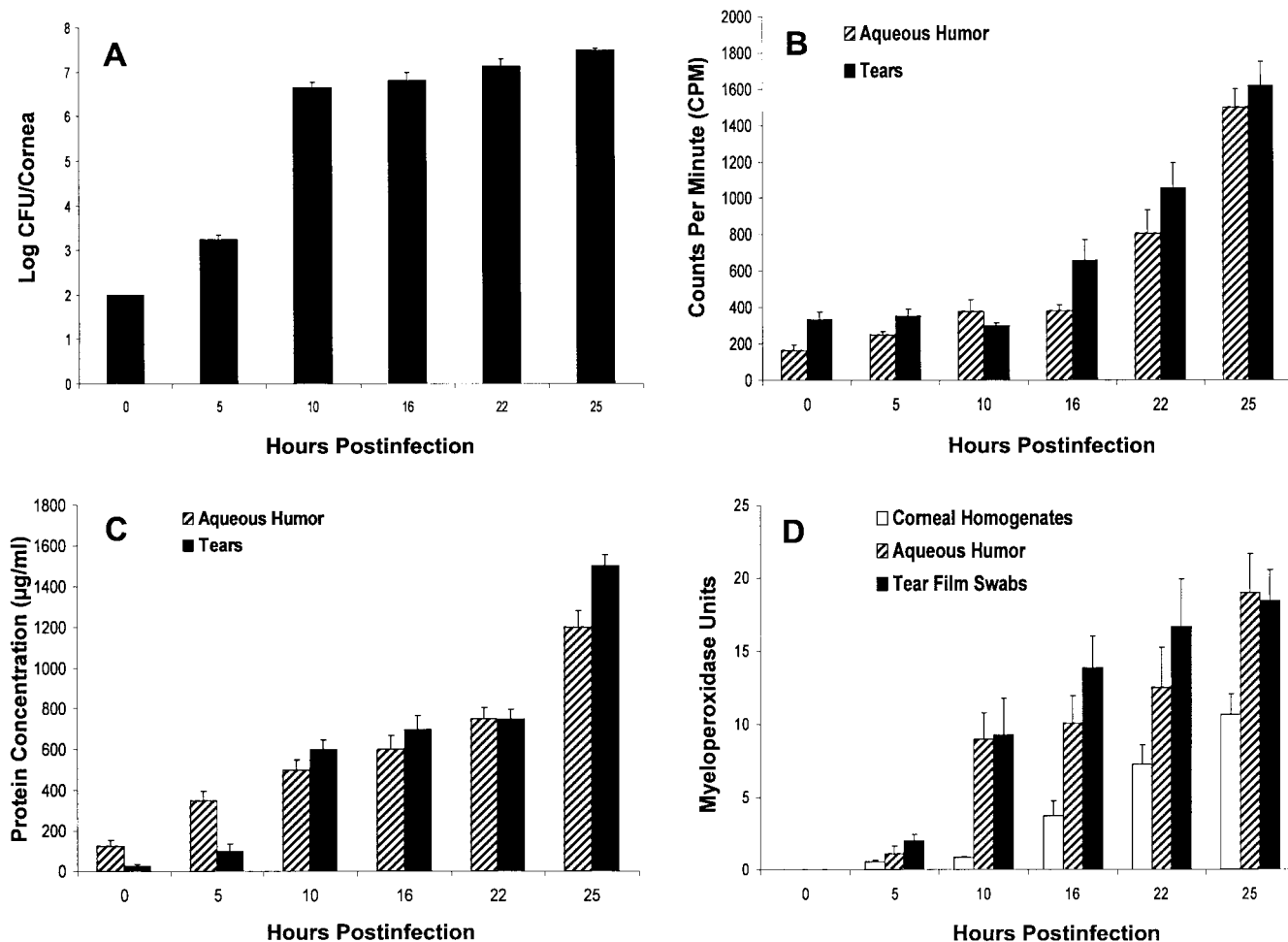
### Effects of Keratitis on PLA<sub>2</sub> Activity, MPO Activity, and Protein Content

The replication of *Staphylococcus* in rabbit corneas ( $n = 6$  rabbits/group) reached 3 log CFU/cornea by 5 hours PI, nearly 7 log CFU/cornea by 16 hours PI, and maintained just over 7 log CFU/cornea up to 25 hours PI (Fig. 3A).

The PLA<sub>2</sub> activity of tears or aqueous humor collected from infected rabbits ( $n = 6$  rabbits/group) in cleaving arachidonic



**FIGURE 2.** Effect of the sleep cycle on PLA<sub>2</sub> activity in rabbit tears. The counts per minute released when <sup>14</sup>C-labeled *Staphylococcus* was incubated with tears collected from rabbits ( $n = 6$ ) at six different times during the day or night. Data are expressed as the mean  $\pm$  SEM.



**FIGURE 3.** Effects of keratitis on PLA<sub>2</sub> activity, MPO activity, and protein content in ocular tissues. (A) Colony-forming units per cornea in rabbit eyes after intrastromal injection of *S. aureus* 8325-4 (100 CFU). Data are expressed as the mean  $\pm$  SEM. (B) The counts per minute released when <sup>14</sup>C-labeled *Staphylococcus* was incubated with aqueous humor and tears collected from rabbits ( $n = 6$ /group) at each time point during infection. Data are expressed as the mean  $\pm$  SEM. (C) Total protein content (in micrograms per milliliter) of aqueous humor and tears collected from rabbits ( $n = 6$ /group) at each time point during infection. Data are expressed as the mean  $\pm$  SEM. (D) MPO activity in corneal homogenates, aqueous humor, and tears collected from rabbits ( $n = 6$ /group) at each time point during infection. One unit of MPO activity was equivalent to approximately 100,000 PMNs. Data are expressed as the mean  $\pm$  SEM.

acid from bacterial membranes was analyzed at 5, 10, 16, 22, and 25 hours PI. PLA<sub>2</sub> activity in the tears of infected rabbit eyes, as measured by counts per minute, increased significantly by 22 hours PI compared with tears of normal rabbits ( $P = 0.0229$ ), and demonstrated a fivefold increase in activity by 25 hours PI ( $P \leq 0.0001$ ; Fig. 3B). PLA<sub>2</sub> activity of infected aqueous humor increased significantly by 22 hours PI ( $P = 0.0027$ ) and demonstrated a ninefold increase in activity by 25 hours PI ( $P \leq 0.0001$ ; Fig. 3B).

Total protein concentration in tears and aqueous humor of *S. aureus*-infected rabbits ( $n = 6$  rabbits/group) collected at 5, 10, 16, 22, and 25 hours PI was determined. Both tears and aqueous humor collected from infected rabbit eyes demonstrated an increase in total protein with progression of infection (Fig. 3C), correlating with the increased PLA<sub>2</sub> activity in these two fluids.

The MPO activity of aqueous humor, ocular swabs, and corneal homogenates collected from each rabbit ( $n = 6$  rabbits/group) at 5, 10, 16, 22, and 25 hours PI was assayed. The MPO activity in aqueous humor, ocular swabs, and corneal homogenates of infected eyes increased from 10 to 25 hours PI. Infected eyes demonstrated a significant increase in MPO activity compared with uninfected eyes beginning at 10 hours PI in the aqueous humor ( $P = 0.03$ ), at 16 hours PI in the

ocular tear film swabs ( $P = 0.0024$ ), and at 22 hours in the corneal homogenates ( $P = 0.0007$ ; Fig. 3D).

## DISCUSSION

This study demonstrates the important role of PLA<sub>2</sub> in killing *S. aureus* in the rabbit tear film and shows that this activity significantly declines with age, increases during sleep, and subsequently declines after periods of sleep. Furthermore, the results show that the PLA<sub>2</sub> activity in the tears and aqueous humor increases in parallel with PMN concentrations (MPO activity) and protein increases in the tissue during *S. aureus* keratitis.

Previous findings by Moreau et al.<sup>14</sup> demonstrated the ability of PLA<sub>2</sub> in rabbit tears to kill *S. aureus*. The data presented herein show the bactericidal role of this enzyme in the tear film and also indicate that the bactericidal activity of this enzyme decreases with the age of the rabbit. The highest bactericidal activity was clearly observed in those tears collected from young rabbits, whereas the bactericidal activity declined significantly in those tears collected from aged rabbits. The results of the bactericidal assay correlated with those results obtained from the radioactive assay specific for PLA<sub>2</sub>, so that enzyme

activity declined significantly with age. Saari et al.<sup>39</sup> demonstrated similar findings with tears collected from human subjects, demonstrating a decrease in PLA<sub>2</sub> concentration that occurred with an increase in age. These findings suggest that loss of this important ocular host defense could render the corneas of aged individuals more susceptible to *S. aureus* infection than the corneas of younger individuals.

The effects of the sleep cycle on the activity of PLA<sub>2</sub> in rabbit tears were also determined. Peaks in PLA<sub>2</sub> activity were noted in tears collected from rabbits in deep sleep, with a steady decline in activity noted as the day progressed, when rabbits were awake and active. These findings correlate with studies that suggest that the composition of the tear film varies between open-eye tears and closed-eye tears.<sup>40</sup> The normal composition of tears that are collected from open eyes is primarily lysozyme, lactoferrin, lipocalin, and secretory IgA (sIgA).<sup>40</sup> However, during sleep, there is a decrease in tear flow, and the concentration of total tear protein increases.<sup>41</sup> The composition of the tear film is altered, so that sIgA concentrations increase, serum proteins (tear albumin, fibronectin, and complement) increase, and there is a large recruitment of PMNs to the tear film.<sup>40-43</sup> Because neutrophils are an established source of PLA<sub>2</sub>,<sup>44,45</sup> their influx into the tear film could serve as an important source of PLA<sub>2</sub> during sleep.

The role of PLA<sub>2</sub> was also analyzed during the progression of *S. aureus* keratitis. Activity of PLA<sub>2</sub> increased substantially, as measured in the aqueous humor and tear film collected from rabbits as the infection progressed. Increased PLA<sub>2</sub> activity also correlated with an increase in total protein concentration in these two fluids, although some of the increases in total protein could be due to increased colony-forming units at the later time points. Inflammation that is characteristic of keratitis in the rabbit model includes chemosis, injection, and accumulation of pus on the corneal surface.<sup>34,46</sup> Soluble bacterial products, such as toxins,<sup>47</sup> mediate tissue damage and induce host chemotactic agents, such as complement and cytokines, that are known mediators of ocular inflammation.<sup>47,48</sup> In addition, inflammation may be due in part to the release of arachidonic acid after digestion of the bacterial inoculum by PLA<sub>2</sub>.

The increase in PLA<sub>2</sub> activity in infected rabbit eyes probably reflects a common host response to changes in the ocular environment. Song et al.<sup>49</sup> have demonstrated increased PLA<sub>2</sub> activity in the eyes of patients with chronic blepharitis. Tears collected from patients with blepharitis contained twice the PLA<sub>2</sub> activity of those of normal individuals.<sup>49</sup> In addition, Aho et al.<sup>50</sup> found a similar two-fold increase in PLA<sub>2</sub> activity in the eyes of patients with keratoconjunctivitis sicca.

The parallel increase in MPO and PLA<sub>2</sub> activity during infection suggests that PMNs are an important source of PLA<sub>2</sub> in the tear film, particularly in response to *S. aureus* keratitis. Sloop et al.<sup>36</sup> have demonstrated that neutrophils in the tear film are derived in part from the eyelid, and that the eyelid-derived PMNs far exceed those that migrate into the cornea from the limbus during *Staphylococcus* keratitis. The present study confirms this point, in that the MPO activity of the tear film was several times higher than that of the corresponding corneal homogenate. These results are also supported by other studies that have demonstrated that neutrophils from the tear film are involved in inflammation of the cornea.<sup>36,51,52</sup> In addition, one mechanism proposed by Song et al.<sup>49</sup> for the increased PLA<sub>2</sub> activity observed in patients with chronic blepharitis suggests that PMNs responding to bacterial inflammatory factors migrate into the tear film and then release quantities of PLA<sub>2</sub>.

In summary, this study has demonstrated factors that affect the host defense of the ocular tear film by altering the activity of phospholipase A<sub>2</sub>. Factors that affect host defense include age and the sleep cycle, as well as the progression of keratitis mediated by *S. aureus*.

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