Secretory IgA Inhibits *Pseudomonas aeruginosa* Binding to Cornea and Protects Against Keratitis

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**Purpose.** To determine whether secretory IgA (SIgA) antibody inhibits *Pseudomonas aeruginosa* binding to cornea in vitro and if boosting SIgA antibody in tears using heat-killed *P. aeruginosa* as an immunizing antigen is protective in vivo in experimentally induced bacterial keratitis in the mouse.

**Methods.** SIgA, immunoglobulin-G, immunoglobulin-M, and an undiluted crude human milk preparation were tested in vitro for their ability to inhibit *P. aeruginosa* binding to the scarified corneas of adult (6 weeks to 6 months of age) mice by topical application of each before similar delivery of the bacterial inoculum. Scanning electron microscopy (scanning EM) was used to quantitate bacterial adherence. In vivo mice were immunized topically with heat-killed *P. aeruginosa* or sham immunized by application of a similar volume of phosphate-buffered saline (PBS). Tears were collected from both groups of mice and levels of immunoglobulins (Igs) measured by enzyme-linked immunosorbent assay (ELISA). After the second immunization, the same two groups were challenged ocularly with 5.0 X 10^7 colony forming units *P. aeruginosa* and the response to infection graded.

**Results.** In vitro, after a 30-minute preincubation with Igs, SIgA (250 µg/ml) significantly decreased *P. aeruginosa* binding to cornea in vitro when compared to the number of bacteria bound in PBS control specimens, and binding reduction was concentration dependent. In vivo, 15 days after a second ocular topical immunization, tear SIgA was elevated significantly and was specific for *P. aeruginosa* when measured by ELISA. In vivo, corneal disease response grades in the heat-killed antigen immunized mice also were significantly less severe when compared to sham-immunized mice.

**Conclusions.** SIgA significantly inhibits binding of *P. aeruginosa* to the wounded mouse cornea in vitro, and inhibition is concentration dependent. In vivo, specific antipseudomonal SIgA in mouse tears can be elicited by topical ocular immunization with heat-killed *P. aeruginosa*, and a significant number of immunized animals with elevated levels of SIgA in their tears exhibited less severe ocular disease after bacterial challenge. Invest Ophthalmol Vis Sci. 1997;38:910–918.

*Pseudomonas aeruginosa* is an opportunistic pathogen capable of producing a rapid, highly destructive corneal infection, often leading to corneal perforation and blindness within 24 to 48 hours after onset.1,2 Adherence to host corneal tissue generally is recognized as an important first step in the pathogenesis of this infection3,4; however, the precise mechanisms used by the bacteria to adhere, colonize, and invade the corneal surface are not yet characterized fully. Studies of *P. aeruginosa* adhesion using the mouse cornea have shown that bacterial binding is influenced by animal age.5–9 Corneal scarification is required for binding in postweaning, 16 postnatal day (P) or older mice,5,9 but is not required for binding to the corneas of immature (P 5) animals.5,9 These studies suggest that components of the ocular surface such as immunoglobulins (Igs), which are present at the corneal surface of the more mature, but undetectable at the.

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surface of the eye of the immature animal,10,11 may function as a host defense mechanism to prevent or modulate bacterial adhesion, invasion, and infectivity.

Immunoglobulin, specifically immunoglobulin-A (IgA), has been implicated as a major contributor to host defense against bacterial infection at mucosal surfaces. Although IgA is thought to act primarily as an immunologic barrier, preventing adherence and adsorption of microbes,12 it can also neutralize microbial virulence factors. Because little is known about the functional role of S IgA at the ocular surface, this study was initiated to determine whether exogenous application of the Ig in vitro modulated Pseudomonas aeruginosa adherence to cornea, whether immunization with heat-killed bacterial antigen increased the level of S IgA in mouse tears, and whether immunized animals were protected against in vivo experimental ocular bacterial challenge.

MATERIALS AND METHODS

Mice

Female Swiss (ICR) mice purchased from Harlan Sprague-Dawley (Indianapolis, IN), or male and female β2m−/− mice, a transgenic knockout mouse that is homozygous for the null allele of the β (2) microglobulin gene and reported to possess low numbers (0.5%) of CD8+ T lymphocytes,10,21 were used (adherence studies only) in the studies described below. All mice were housed in clear plastic cages and fed laboratory rodent chow (Ralston-Purina, St. Louis, MO) and sterilized, prepared culture wells containing 2 to 3 ml sterileized, prepared culture wells containing 2 to 3 ml minimal essential medium with Earle’s salts, L-glutamine, and nonessential amino acids without sodium bicarbonate, pH 7.3 (Gibco) at 37°C. The corneal surface, oriented superiorly in the well, was covered by a thin layer of minimal essential medium.

Bacterial Cell Cultures

Stock cultures of P. aeruginosa, American Type Culture Collection (ATCC) 19660, stored at 25°C on tryptose agar slants (Difco Laboratories, Detroit, MI) were used for inoculation of 60 ml broth medium containing 5% peptone and 0.25% tryptose soy broth (BBL Microbiology Systems, Cockeysville, MD) as described previously. The culture was hemolytic, proteolytic, and, under appropriate conditions, produces lecithinase and exotoxin A. Cultures were grown on a rotary shaker at 37°C for 18 hours, centrifuged at 7000 × g for 20 minutes at 4°C, and diluted with saline (Travenol Laboratories, Deerfield, IL) to a concentration of 2.0 × 10⁷ colony forming units (cfu) per ml, using a standard curve relating viable counts to optical density at 440 nm.22,23 An inoculum of 5 μl containing 5.0 × 10⁷ organisms was used in each of the experiments.

Culture Wells

The culture system used for this study has been described in detail previously.6 Briefly, cell culture dishes (Gibco, Grand Island, NY) 35 × 10 mm and rubber tubing (Norton Plastics, Akron, OH) of 3/16 and 2/16 inch diameters were obtained. The tubing was cut and assembled to facilitate resting of the posterior part of the eye on the 3/16-inch tubing. Locating the optic nerve down inside the 2/16 inch tubing provided greater stability and a superior orientation of the cornea within the 3/16-inch tubing. The entire tubing apparatus was superglued to the bottom of each culture dish and placed under ultraviolet light overnight for sterilization before each experimental use.

Organ Culture. The methods for organ culture6 and scarification of mouse cornea6 have been described previously and were done similarly. In brief, adult Swiss ICR mice, 6 to 8 weeks of age, or β2m−/− mice, 2 to 6 months of age were, anesthetized with isoflurane (Aerrane; Anaquest, Madison, WI) and killed immediately by cervical dislocation. Then animals were placed beneath a 40X stereoscopic microscope, and each eye was observed for corneal clarity before making three 1-mm incisions with a sterile 25-27-gauge needle to the center of the right and left cornea. After this, eyes were enucleated with a sterile scissors and put into sterilized, prepared culture wells containing 2 to 3 ml minimal essential medium with Earle’s salts, L-glutamine, and nonessential amino acids without sodium bicarbonate, pH 7.3 (Gibco) at 37°C. The corneal surface, oriented superiorly in the well, was covered by a thin layer of minimal essential medium.

Adherence Assay

A 5-μl bacterial cell suspension containing 5.0 × 10⁷ cfu P. aeruginosa ATCC 19660 was delivered topically onto the surface of each eye in culture using a calibrated micropipette (Coe-Parmer, Chicago, IL) with a sterile, disposable tip. All eyes immediately were placed in a water-jacketed CO₂ incubator (American Scientific Products, McGaw Park, IL) at 37°C and 5% CO₂ for 60 minutes. Then eyes were rinsed vigorously.
in phosphate-buffered saline (PBS), fixed, dehydrated, and critical-point dried for scanning EM observation. To control for expected individual experimental variation in number of bacteria bound to cornea, a PBS control group was included for each adherence assay.

Specimen Preparation for Scanning Electron Microscopy

After incubation of the organ cultured eyes with the bacteria, the specimens were rinsed vigorously with PBS (pH 7.5) to dislodge nonadherent organisms and fixed immediately at 4°C in a 1:1:1 (vol/vol) fixative containing 2.0% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M phosphate buffer (pH 7.5) for a total of 3 hours, with a change to freshly prepared fixative after 1.5 hours. Then eyes were dehydrated through a graded series of ethanols to 100% and critical-point dried in an AutoSamdri critical-point dryer (Tousimis Research Laboratories, Rockville, MD) with liquid CO₂ as the transition fluid. Eyes with corneas oriented superiorly were mounted on aluminum-specimen stubs with colloidal silver paint suspended in methanol (Tousimis). A thin layer of gold (approximately 30 nm) was evaporated onto the surface of each specimen with a Pelco SC5 sputter coater (Ted Pella, Redding, CA). Eyes were viewed with a JEOL JSM-840A scanning EM (JEOL, Tokyo, Japan) at the settings described below. This procedure also has been used in past studies.⁵

Quantitation of Adherence

Adherent bacteria were quantitated at 60 minutes after their ocular application using scanning EM procedures described previously.⁵,⁷,¹⁹,²⁵ In brief, the number of organisms in a microscopic field of a fixed size was observed. The field size was fixed by using a working distance of 25 mm, fixing the angle from which the sample was viewed (30°) and using a uniform magnification of 3000×. For each test, five fields selected randomly were photographed from each group consisting of three eyes (N = 15) and the negatives photographically enlarged to a total magnification of 6000×. Counts were done with a square measuring 80 mm² at 6000× and the data expressed as the mean number of bacteria bound per cornea. This method of quantitation previously has been shown to parallel results obtained from an alternate method of assay using microbiologic culture of eyes.⁵

Immunoglobulin Treatment In Vitro

Clarified human milk pooled from multiple donors, purified SIgA from pooled milk donors, immunoglobulin-G (IgG) purified from the serum of a single donor, and immunoglobulin-M (IgM) purified from human serum (Cappel, Durham, NC) were used in bacterial adherence inhibition assays. All of the Igs were diluted with PBS to a concentration of 250 μg/ml. The crude milk preparation when tested by sandwich enzyme-linked immunosorbent assay (ELISA), contained 3.0 μg/ml IgM, 2.5 μg/ml IgG, and 4.6 μg/ml IgA, and was used undiluted. These Igs as well as the crude milk preparation also were tested at a concentration of 250 μg/ml or undiluted (crude milk) for the presence of specific anti-Pseudomonas antibody. The methods are described below and were similar, except that the secondary antibody for this system was a goat anti-human mixture of IgG, A and M (Zymed, San Francisco, CA). All of the samples tested positively at the concentrations used. To test whether binding inhibition was concentration dependent, SIgA was used at dilutions of 125 and 62.5 μg/ml. Scarified mouse eyes in organ culture were incubated with either PBS (control), one of the above Igs, or the undiluted crude milk preparation for 30 minutes at 37°C before topical application of the bacteria.

Preparation of Bacterial Antigen for Immunization

A stock culture of P. aeruginosa ATCC 19660 at a concentration of 2.0 × 10⁹ cfu was heat-killed at 55°C for 60 minutes. Culture viability was tested by inoculating a culture dish containing Pseudomonas isolation agar (Difco) and monitoring it for colony growth. No growth was observed after 24 hours at 37°C. The heat-killed stock culture was divided into 1-ml aliquots and frozen at −70°C until used.

Immunization

Swiss ICR mice (N = 20) were ocular topically immunized to stimulate tear SIgA essentially as described previously for rats by Peppard et al. In brief, mice were anesthetized lightly with isoflurane, and a 5-μl aliquot of the heat-killed bacterial suspension was delivered topically to the surface of each eye. This procedure was repeated for 3 consecutive days with a total antigen dose of 6.0 × 10⁷ cfu/mouse. This procedure was repeated 2 weeks after the initial priming immunization, then again 30 days later. Control mice (N = 17) were sham immunized similarly with sterile PBS.

Tear Collection

In the afternoon from approximately 1 to 4 PM, on days 11 and 15 after the first and second immunization, respectively, tears from immunized and control mice were collected and pooled on ice in snap cap microcentrifuge tubes to prevent evaporation as described previously. In brief, to collect tears, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (1:12 dilution of 65 mg/ml stock solution, administered at 0.01 ml/g body weight). Un-
stimulated tears were collected by gentle aspiration with a 5 µl glass capillary tube with a smooth, fire polished tip. Approximately 1 to 1.5 µl of tears can be collected per mouse per time by this technique. The pooled mouse tears from each group were centrifuged at 1800 x g at 4°C to remove particulate debris and frozen at −20°C until tested by ELISA.

Sandwich Enzyme-Linked Immunosorbent Assay

Standard curves for each Ig were established using mouse myeloma protein standards in a sandwich ELISA assay as described earlier. Briefly, wells of Falcon microtest III flexible assay plates (Becton Dickinson, Oxnard, CA) were coated overnight at 4°C with 100 µl/well of the appropriate antiserum: goat antimumouse (GAM) IgA 1:2500, GAM IgM 1:4000 (both from Cappel), GAM IgG$_{total}$ 1:2000 (Accurate Chemicals, Westbury, NY). Plates were washed 5 times 4 minutes each at room temperature (RT) with PBS-T-N (0.05% Tween 20, 0.1% sodium azide), pH 7.4. Myeloma proteins TEPC 15 (IgA), TEPC 183 (IgM), and UCP 10 (IgG$_{total}$) (all purchased from Litton Bionetics, Charleston, SC) were diluted serially, added to each well (100 µl/well), and incubated for 2 hours at 37°C at various concentrations ranging from 50 to 0.39 ng. An irrelevant myeloma protein, IgG$_{s}$, at similar nanogram concentrations was added to control wells. The plate was washed again three times, 4 minutes each in PBS-T-N at RT. Each well was incubated for 2 hours at 37°C with 100 µl/well of a 1:100 dilution of alkaline phosphatase-conjugated GAM Ig(G+A+M, Cappel). After washing a final three times, 4 minutes each with PBS-T-N at RT, 200 µl Sigma 104 phosphate solution (Sigma Chemical, St. Louis, MO) was added to each well and incubated 45 minutes at 37°C. To stop the reaction, 40 µl 3 M sodium hydroxide was added to each well. Absorbance was read at 405 nm on a Titertek Miniskan microplate reader (Flow Laboratories, Mclean, VA), and standard curves were computer generated using a Macintosh SE equipped with Cricket graph 1.3 statistical software (Abacus Concepts, Berkeley, CA). Collected mouse tear samples at appropriate dilutions (IgA 1:4000, IgM 1:1000, IgG$_{total}$ 1:50) then were tested using this system, and the concentration of each Ig was calculated using the standard curves. Mouse tears also were tested to determine if the IgA in the tears (tested for as described above) consisted of SIgA and whether it was specific against P. aeruginosa by use of a similar ELISA assay system. For these assays, each test well was coated with either a polyclonal antibody specific for rat SC at a concentration of 5 µg/ml or a 10$^6$ dilution of a heat-killed P. aeruginosa preparation overnight at 4°C. The plates were washed two times with PBS-T-N at RT and then tears diluted 1:4000 in PBS were added to each well (100 µl/well) and incubated at 37°C for 2 hours. The plates were washed again four times with PBS-T-N at RT and a second antibody, goat antimumouse IgA conjugated to biotin, was added to each well at a concentration of 1 µg/ml. After a 2-hour incubation at 37°C, the plates were washed again six times with PBS-T-N, and avidin–peroxidase (1:400) was added and the plates incubated for 30 minutes at RT. After a final three washes, the plates were developed with ABTS (2,2'-Azino-bis[3 ethylbenzthiazoline]-6-sulfonic acid) and read spectrophotometrically as described above.

Infection

In two separate experiments, mice were infected either 12 or 19 days after the second immunization with similar outcome. To infect animals, antigen and sham-immunized Swiss ICR mice were anesthetized with isoflurane, placed beneath a stereoscopic microscope, and the surface of the left eye was scarified as described above. A 5-µl bacterial suspension containing 5.0 x 10$^7$ cfu P. aeruginosa strain ATCC 19660 was delivered topically to the surface of the wounded cornea.

The ocular response of mice to P. aeruginosa infection was evaluated macroscopically and graded at 24 hours and then every day for a total of 2 weeks after infection in a double-masked fashion. The response to infection was graded$^{24}$ using a scale of 0 to +4, with 0 = clear or slight opacity partially covering the pupil, +1 = slight opacity fully covering the anterior segment, +2 = dense opacity, partially or fully covering the pupil, +3 = dense opacity covering the entire anterior segment, or +4 = corneal perforation or phthisis. A mean clinical score was calculated by summation of the scores for each group of mice divided by the total number of mice scored at each timepoint. Eyelids that were fused shut when graded were not scored. No attempt was made to propote the eye in such mice because this can mechanically induce perforation.

Statistical Analysis

An unpaired, two-group Student's t-test using a two-tailed hypothesis was used to test for significance of the scanning EM data, and a chi-square analysis with no Yates continuity correction was used to test for the significance of the differences in corneal disease response using a P ≤ 0.05 confidence interval for both.

In Vitro Agglutination

To test for in vitro agglutination of P. aeruginosa by each of the Igs (SIgA, IgM, or IgG) or crude human milk, 0.1 ml Igs at 250, 125, or 62.5 µg/ml, and crude human milk undiluted, diluted 1:1, or 1:2 was added to 0.1 ml of an overnight culture of P. aeruginosa strain ATCC
FIGURE 1. Scanning electron microscopy analysis of the effects on bacterial binding of immunoglobulins (250 μg/ml) or crude human milk applied topically to mouse scarified cornea 30 minutes before application of the bacterial inoculum. Two separate experiments were run, each with its own phosphate-buffered saline control. Secretory immunoglobulin-A (250 μg/ml) significantly (P< 0.0001) reduced bacterial binding to cornea.

19660 and incubated in Falcon microtest III flat-bottom flexible assay plates (Becton Dickinson). An irrelevant MAb, anti-HLA DR5 (ATCC hybridoma SFR3-DR5) at similar concentrations and PBS were both used as irrelevant control specimens. Agglutination was not detected visually for any of the Igs or crude human milk after 2 to 5 hours of incubation at RT.

RESULTS

Immunoglobulin Treatment
Undiluted human milk, SlgA, IgM, or IgG, the latter Igs each at 250 μg/ml concentration, were applied topically to the scarified corneas of eyes enucleated from 6- to 8-week-old Swiss ICR mice. Eyes were incubated in organ culture for 30 minutes at 37°C, after which an inoculum of 5.0 × 10^7 cfu bacteria was applied topically and the eyes incubated at 37°C for an additional 60 minutes. Significant inhibition of bacterial adherence was obtained only by topical application of SlgA (59%; P ≤ 0.0001) before addition of the bacterial inoculum, and these data are presented in Figure 1. The experiment was repeated using 2- to 6-month-old βm^6 mice, and similar results were obtained. These mice are bred in-house, and a group was tested similarly to determine whether the binding inhibition observed in Swiss ICR animals was a general phenomenon and not one confined to the outbred animal. Because the knockout mice tested similarly to the Swiss ICR animals, data obtained from the latter experiment were combined with the first for statistical analysis. Next, to determine if the observed binding inhibition was concentration dependent, SlgA was diluted to a concentration of either 125 or 62.5 μg/ml, applied topically to scarified eyes, and the eyes incubated at 37°C in vitro for 30 minutes. Control mice were treated similarly, but received PBS topically instead of antibody. The bacteria then were applied topically, and the eyes were incubated for an additional 60 minutes at 37°C. Only at a concentration of 125 μg/ml was significant (18%, P ≤ 0.0002) inhibition of adherence obtained. This inhibitory effect also significantly was less effective than the inhibition produced by the 250 μg/ml concentration of SlgA. SlgA at a concentration of 62.5 μg/ml showed only slight reduction of bacterial binding (2% to 7%), which did not differ significantly from the number of bacteria bound in control specimens. These data concerning concentration dependency are presented in Figure 2.

Enzyme-Linked Immunosorbent Assay of Mouse Tears
Adult, 6 to 8 weeks of age, Swiss ICR mice were ocular-topically immunized with heat-killed P. aeruginosa specifically to elevate the concentration of SlgA in tears. Tears were collected at two selected timepoints to provide a sufficient amount of sample for ELISA testing. These data are shown in Figure 3. At 2 weeks after the initial topical ocular challenge with the heat-killed organisms, the amount of IgA in collected tears from immunized mice was elevated slightly (23%) when compared with the amount of this Ig in tears collected from PBS sham-immunized mice. A slight increase in
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FIGURE 3. Enzyme-linked immunosorbent assay of tears collected from control (C) and immunized (E) mice. The level of immunoglobulin-A (IgA) detected in tears was elevated by 23% in mice 2 weeks after the first immunization with heat-killed bacteria when compared to mice that were sham immunized with phosphate-buffered saline. The level of IgA was elevated approximately 197% in tears of immunized experimental mice compared to the levels detected in sham-immunized control mice 15 days after the second immunization. The level of immunoglobulin-M and IgG2a also was elevated at these times; however, the total concentration of each of these immunoglobulins was approximately 90% to 98% less than IgA.

FIGURE 4. Effect of immunization of mice against corneal disease. Comparison of the incidence of a grade 0/ +1 response versus all other ocular responses was performed by chi-square analysis with no Yate's continuity correction. This test showed that mice immunized with heat-killed organisms (N = 20) and subsequently challenged with 5.0 × 10^7 colony forming unit *Pseudomonas aeruginosa* were protected significantly (P = 0.001) at all periods after 24 hours when compared to similarly challenged phosphate-buffered saline sham-immunized (N = 17) mice.

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In Vivo Response to Ocular Challenge With *Pseudomonas aeruginosa*

Mice that were ocular-topically immunized with heat-killed *P. aeruginosa* before experimental ocular challenge with viable organisms exhibited significantly less-severe corneal disease than did PBS sham-immunized mice. As shown in Figure 4, the response to ocular infection was similar at 24 hours postchallenge in both immunized and sham-immunized animals. However, at all other timepoints, the disease response grade was significantly (P = 0.0001) less severe in the immunized group of mice. With time, corneal disease severity progressed in both groups, and this was reflected by the numeric increase in recorded grade response. Nonetheless, disease severity remained significantly less in the immunized versus control mice (P < 0.001) at all times examined. By day 14 after infection, 7 (35%) of 20 of the immunized mice had restored corneal clarity (grade 0/ +1), whereas only 1 (5%) of 17 of the sham-immunized mice had this response grade.

DISCUSSION

Previous work in this laboratory localized Igs at the surface of the P 37 mouse cornea using Sternberger's sensitive peroxidase antiperoxidase technique. Data from that study showed IgA, IgG1, IgG2a, and IgM at the surface of the normal corneal epithelium, and these Igs were associated intimately with microvilli of surface epithelial cells. Further work established the kinetics of Ig appearance during postnatal development in the mouse. One of these Igs, IgA, was localized initially at the ocular surface of the P 21 aged mouse. This period previously had been described as transitional from ocular susceptibility to the organism, observed in younger mice, to resistance and eventual clearing of the bacterial infection by 3 to 4 weeks, as observed in P 37 outbred mice. The possible role that...
IgA or other Igs might play in these events was not investigated.

The current study shows that purified SfIA from human milk significantly decreases P. aeruginosa binding to the mouse scarified corneal epithelium in an in vitro organ culture model. The biologic relevance of these data also was tested in an in vivo experimental ocular infection mouse model. Animals that were ocular-topically immunized with heat-killed P. aeruginosa showed that this immunization protocol enhanced the level of tear IgA by approximately 197% compared to control levels. In addition, in vivo ocular infection of animals with increased levels of tear IgA resulted in less-severe ocular disease when compared with the response of mice that had been sham immunized. These data provide strong evidence that increasing the level of tear IgA protects against experimentally induced bacterial infection. The mechanisms by which in vivo protection is mediated are not clear.

IgA antibodies may prevent bacterial colonization by specific interactions with bacterial adhesins required for binding to mucosal tissues. In this regard, preliminary ELISA data (Hazlett et al, unpublished data, 1995) showed that tear SfIA interacts positively with several outer membrane-associated bacterial structures, including pilus, flagellum, and lipopolysaccharide of strain 19660. Hypothetically, inhibiting the number of organisms bound to the ocular surface would be advantageous, because this might diminish the host inflammatory cell response to eliminate bacteria from the cornea, in turn leading to less "innocent bystander" destruction of corneal tissue. IgA antibodies also may prevent colonization by promoting opsonization or agglutination of bacteria. With respect to the latter possibility, several laboratories have shown that human salivary SfIA antibodies function to agglutinate various streptococcal strains29-30 to prevent mucosal colonization. Further, Biesbrock et al34 suggested that complexes consisting of low molecular weight mucins and SfIA are more efficient at inducing agglutination than uncomplexed SfIA. In an in vitro model of adherence of Salmonella typhimurium to cultured Madin-Darby Canine Kidney cells, a specific IgA antibody also reduced binding of S. typhimurium by agglutination.19 In the current study, we did not detect agglutination of P. aeruginosa strain 19660, even when incubating the bacteria with 250 μg/ml of purified SfIA. This concentration was greater than required to agglutinate bacteria effectively in other nonocular systems10,19,29,39 which used concentrations ranging from 0.1 to 20 μg/ml antibody.

In addition to IgA, a protective role for other Ig classes also has been suggested. All three major human Ig classes, IgA, IgM, and IgG, specific for P. aeruginosa lipopolysaccharide opsonized P. aeruginosa for in vitro phagocytic killing and protected neutropenic mice in vivo, indicating the potential for each as a passive therapeutic agent.26 In patients with urinary tract infections, high amounts of IgG as well as IgA are detected in urine and can prevent reinfection by the same strain of bacteria, suggesting the involvement of specific antibody interactions.52 The mechanism for this is not known, but antibody-mediated steric hindrance may be of importance.53 Like IgA, IgM is transported actively into the secretions of IgA-deficient individuals and may protect in a similar manner.53 Because other Ig classes protect host tissue from bacterial adherence, we also tested for two Igs (IgM and Ig2a) previously detected immunocytochemically at the mouse ocular surface.40 Our findings were consistent with previous reports showing that SfIA is present in normal tears in substantial amounts, with little IgM or IgG.26 Neither IgG, IgM, or an undiluted crude human milk preparation was able to reduce significantly the number of bacteria adhering to wounded mouse cornea in vitro. Nor did immunization substantially increase levels of these Igs in tears when compared with IgA, suggesting that they contribute little in protection against keratitis in this model.

Milk, or more specifically colostrum, also has been shown to protect the gastrointestinal epithelium from bacterial colonization. This effect has been attributed to its SfIA antibody content, but other components, such as glycoconjugates, also may be contributory. Giugliano et al34 used either trypsin or sodium metaperiodate treatment of a 50% ammonium sulfate precipitation product obtained from human milk for binding inhibition studies. Trypsin-treated product increased the inhibition titer, whereas sodium metaperiodate treatment, which oxidizes carbohydrates mildly without digestion of core proteins, abolished the inhibitory effects of the precipitated milk product.39 One glycoconjugate-containing glycoprotein that has been targeted specifically as an inhibitory factor in mucosal secretions is SC, a glycoprotein of approximately 83 KDa, which is approximately 18% to 22% carbohydrate by weight.18,38 Dallas and Rolfe34 reported that free SC inhibited the binding of Clostridium difficile to the gastrointestinal epithelium in vitro by preventing toxin A receptor binding. In the current study, we tested tears for the presence of IgA and SfIA by ELISA. In the latter case, availability of an anti-SC polyclonal antibody allowed us to confirm that the IgA in collected tears detected by ELISA was SfIA. Nonetheless, this assay did not allow us to distinguish between bound versus free SC, which can be present in amounts equal to those of IgA-bound SC.35 In this regard, hypothetically during an infection, or after topical application of bacteria onto the eye in culture, bacterial glycosidases could release oligosaccharides from SfIA and free SC. Although this possibility awaits
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Experimental confirmation, oligosaccharides may be of relevance to binding inhibition, because previous reports from this laboratory and others have shown that sugars such as mannose and sialic acid are constituents of the *P. aeruginosa* pilus receptor. It is reasonable to suggest that if carbohydrate components are released, binding inhibition could result.

SIgA functions in a milieu that contains innate humoral defense factors such as lysozyme, lactoferrin, and lactoperoxidase. The antiinfectious activity of these factors is, in certain models, potentiated by SIgA. Examination of bacterial-mediated oligosaccharide release, as well as analysis of tears from immunized mice for the presence of other possible protective factors, is required to define more precisely the role SIgA plays in protection of the eye in bacterial infections. The potential applicability of such a simple immunization protocol using heat-killed bacterial antigen may offer an attractive alternative to current therapy for *P. aeruginosa* keratitis.

**Key Words**
immunization, keratitis, protection, *Pseudomonas aeruginosa*, SIgA

**References**

27. Wells PA, Hazlett LD. Quantitation of immunoglobulin...
34. Dallas SD, Rolfe RD. Inhibition of Clostridium difficile toxin A receptor binding by human milk secretory component. Presented at the 95th General Meeting of the American Society for Microbiology. Abstract E-28, 1995, 25.