Corneal Antibody Levels to Ribitol Teichoic Acid in Rabbits Immunized With Staphylococcal Antigens Using Various Routes

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Although *Staphylococcus aureus* is an important cause of infectious diseases of the eye and hypersensitivity lesions of the cornea, little is known about ocular immunity to this pathogen. Using an enzyme-linked immunosorbent assay, we measured antibody titers to ribitol teichoic acid, the major antigenic determinant of *S. aureus*, in corneas as well as serum and tears after immunizing rabbits using the following routes: intradermal injection of cell wall mixed with complete Freund's adjuvant, subconjunctival injection of cell wall mixed with complete Freund's adjuvant, topical application of cell wall to the eye or topical application of viable *S. aureus* to the eye. IgG titers to ribitol teichoic acid were found consistently in corneas after intradermal and subconjunctival immunization with cell wall and topical immunization with viable *S. aureus*. After intradermal immunization with cell wall, IgG titers in cornea were higher than tears but lower than serum, which was presumably the source of the IgG antibodies for the cornea. After subconjunctival immunization with cell wall or topical immunization with viable *S. aureus*, IgG titers in corneas were higher than tears and generally higher than serum, suggesting that the ocular tissues were a local source of IgG. On the other hand, IgA titers to ribitol teichoic acid were found in tears but not in serum and were found only occasionally in corneas, suggesting that IgG responses to staphylococcal antigens may be more important than IgA responses in the cornea. The results of this study suggest that corneal antibodies to ribitol teichoic acid may be influenced by exposure to staphylococcal antigens not only in the external eye but also at sites remote from the eye. Invest Ophthalmol Vis Sci 28:1553-1558, 1987

*Staphylococcus aureus* occupies an important position in bacterial diseases of the eye. *S. aureus* is one of the most common causes of chronic conjunctivitis, blepharitis, corneal ulcers, acute and chronic dacryocystitis, orbital cellulitis and endophthalmitis. Moreover, hypersensitivity to *S. aureus* may be responsible for corneal phlyctenules and catarrhal infiltrates and contribute to staphylococcal blepharitis.

The only serologic tests of major clinical importance in the diagnosis of staphylococcal disease are assays for antibodies to ribitol teichoic acid, the major antigenic determinant of *S. aureus*. These assays have proven useful not only in diagnosing but also in assessing response to therapy and relapses of patients with staphylococcal diseases including bacteremia with metastatic abscesses, endocarditis with negative blood cultures because of prior therapy, and deep-tissue infections inaccessible to culturing. Additionally, we found that serum antibody levels to ribitol teichoic acid correlated with the development of corneal phlyctenules, catarrhal infiltrates and blepharitis in rabbits after immunization by intradermal injections of cell wall or intravenous injections of ribitol teichoic acid and prolonged topical challenge with viable *S. aureus*. Because of the importance of antibodies to ribitol teichoic acid and because little is known about ocular immunity to *S. aureus*, we measured antibody levels to ribitol teichoic acid in rabbit serum and tears using an enzyme-linked immunosorbent assay (ELISA) after immunization with *S. aureus* antigens using the following routes: intradermal injection of cell wall mixed with complete Freund's adjuvant, subconjunctival injection of cell wall mixed with complete Freund's adjuvant, topical application of cell wall to the eye and topical application of viable *S. aureus* to the eye. We found that all four immunization groups showed an IgG antibody response to ribitol teichoic acid in serum and tears and an IgA antibody response to ribitol teichoic acid in tears but not serum. The results of our study suggested that ocular immune responses to *S. aureus* may be influenced by exposure.
to staphylococcal antigens not only in the external eye but also at sites remote from the eye.

In the present study, we sought to compare the effect of different routes of immunization on antibody levels to ribitol teichoic acid in corneas. Corneal antibody levels to *S. aureus* may be important not only in host defense against this bacteria but also in hypersensitivity lesions of the cornea such as phlyctenules and catarhal infiltrates. There have been no previous studies of corneal antibody levels to *S. aureus* antigens or, for that matter, to any other bacterial antigens after immunization using various routes.

**Materials and Methods**

The strain of *S. aureus* that we used for our cell wall preparation and for topical immunization belonged to phage-type 95 and was coagulase-positive, beta-hemolytic, pigmented and gentamicin sulfate-resistant. Cell wall was prepared as previously described using a cell-fractionator at a pressure of 52,000 psi.6 Our cell wall preparation was treated with deoxyribonuclease and ribonuclease to digest nucleic acids and trypsin to remove protein A. The electron microscopic and chemical analysis of our cell wall preparation (peptidoglycan-ribitol teichoic acid) has been described in detail previously.6 Vancomycin in a final concentration of 0.1% was added to our cell wall preparation to prevent contamination. Cell wall (1 mg/ml) was added one drop at a time to complete Freund's adjuvant (1 mg/ml). A cell wall emulsion (0.5 mg/ml) was prepared in this way using equal volumes of cell wall and complete Freund's adjuvant. Rabbits were immunized with cell wall rather than ribitol teichoic acid because the latter is poorly immunogenic in rabbits even when mixed with complete Freund's adjuvant.10

We used 96 New Zealand white female outbred rabbits weighing approximately 2 kg: 76 experimental and 20 control rabbits. All investigations described in this manuscript conformed to the ARVO Resolution on the Use of Animals in Research. Prior to intradermal and subconjunctival injections, general anesthesia was induced by an intramuscular injection of 2 ml of chlorpromazine hydrochloride (25 mg/ml) followed 1 hr later by an intravenous injection of 3 ml of Equi-Thesin (chloral hydrate 42.5 mg/ml and sodium pentobarbital 10.5 mg/ml) (UCLA Pharmaceutical Services, Los Angeles, CA).

Four immunization groups were used. Rabbits in Group 1 were injected intradermally in each rear footpad with 0.1 ml of cell wall-complete Freund's adjuvant, so that each rabbit received a total of 0.1 mg of antigen. Control rabbits received the same volume of normal saline-complete Freund's adjuvant injected intradermally into the rear footpads. Two weeks later, the injections were repeated in the front footpads, using precisely the same volumes and amounts of cell wall-complete Freund's adjuvant or normal saline-complete Freund's adjuvant. Rabbits in Group 2 received an injection of 0.1 ml of cell wall-complete Freund's adjuvant beneath the conjunctiva of the inferior fornix of each eye so that each rabbit received a total of 0.1 mg of antigen. Control rabbits were injected with the same volume of normal saline-complete Freund's adjuvant in the same location of both eyes. Two weeks later, the injections were repeated beneath the conjunctiva of the superior fornix of both eyes using precisely the same volumes and amounts of cell wall-complete Freund's adjuvant or normal saline-complete Freund's adjuvant.

Rabbits in Group 3 were immunized by topical application of a suspension of viable *S. aureus* in tryptic soy broth (5 × 10⁸ colony-forming units/ml). Rabbits in Group 4 were immunized by topical application of either 0.2 mg/ml or 2 mg/ml cell wall in sterile injectable normal saline. Rabbits undergoing topical immunization received 1 drop (approximately 50 μl) in each eye five times daily for 5 days each week for 12 weeks. Control rabbits for Groups 3 and 4 received topical sterile normal saline, the diluent for the cell wall preparation, or tryptic soy broth, the diluent for viable *S. aureus*, on the same schedule as above.

In all four experimental and control groups, serum and tears were obtained from all rabbits prior to immunization. In Groups 1 and 2 undergoing intradermal or subconjunctival immunization, serum and tears were collected from all rabbits at 4 weeks after the first immunizing injection (which was 2 weeks after the second immunizing injection). Rabbits were sacrificed from both groups at this time to obtain their corneas. Eight weeks after the first immunizing injection, serum and tears were collected from the remaining rabbits, and additional rabbits were sacrificed to obtain corneas. Twelve weeks after the first immunizing injection, serum and tears were collected from the remaining rabbits which were then sacrificed to obtain their corneas.

In Groups 3 and 4 undergoing topical immunization, serum and tears were collected from all rabbits after 4 weeks of topical immunization. Rabbits from both groups were sacrificed at this time to obtain their corneas. After 8 and 12 weeks of topical immunization, serum and tears were collected from the remaining rabbits, and additional rabbits were sacrificed to obtain their corneas.

Arterial blood was drawn from rabbit ears and was centrifuged at 2,000 rpm for 15 min to separate the serum from the clot. Tears were collected patiently
from both eyes of each rabbit by capillary attraction into 5 µl accupette pipettes (Dade Diagnostics, Inc., Aguada, Puerto Rico) as previously described. The pipettes were applied to the lower lid tearfilm meniscus with care taken not to touch the eye to avoid tear stimulation and transudation from conjunctival vessels. To collect enough tears without causing ocular irritation, it was sometimes necessary to collect tears at two different times on the same day. Both ends of the pipettes were sealed with parafilm. The samples from both eyes were pooled so that a total volume of approximately 20 µl was obtained from each rabbit. In Groups 3 and 4, topical applications of cell wall or viable S. aureus were discontinued 72 hr prior to tear collection. Serum and tears were frozen immediately at −70°C.

After sacrifice, the central cornea (10 mm in diameter) of both eyes was excised leaving a 1–2 mm rim of peripheral clear cornea. The excised corneas were rinsed repeatedly with sterile 0.01 M phosphate-buffered saline, pH 7.4, and blot-dried to eliminate any possible contribution from tears, blood or aqueous humor. The corneas from both eyes of each rabbit were minced into small pieces using a #11 scalpel blade, pooled and added to pre-weighed plastic test tubes containing 1 ml of 0.01 M phosphate-buffered saline, pH 7.4. The tubes were weighed again to determine the net weight of corneal tissue to ±0.1 mg. The test tubes were capped tightly, sealed with parafilm and incubated at 37°C in a shaking water bath for 72 hr. After 72 hr, the tubes were centrifuged at 1,000 rpm for 15 min, and the supernatant fluid was then frozen at −70°C. Occasional sections of peripheral and central corneas from each group were placed in neutral-buffered formalin for histopathologic examination.

An ELISA was used to measure antibodies to ribitol teichoic acid in rabbit sera, tears and corneal eluates. Purified ribitol teichoic acid was given to us by Roman Dziarski, PhD, Indiana University, Gary, IN. The preparation and chemical analysis of the purified ribitol teichoic acid used in this study have been detailed elsewhere. Ribitol teichoic acid was dissolved in phosphate-buffered saline (pH 7.4) containing 10% carbonate coating buffer (pH 9.6) at a concentration of 10 µg/ml. Flat bottom microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with 100 µl/well of ribitol teichoic acid. The plates were incubated at 4°C overnight. The plates were washed four times with phosphate-buffered saline-Tween 20, pH 7.4. Eight µl of serum, tears or corneal eluate were added to wells in the plates, and serial dilutions were made in phosphate-buffered saline from 1:25–1:51, 200. The plates were incubated at room temperature for 2 hr and then washed four times with phosphate-buffered saline-Tween 20. To measure IgG antibodies to ribitol teichoic acid, we added 100 µl of 1:1600 goat antirabbit IgG, heavy and light chain specific, conjugated with peroxidase (American Qualex, La Mirada, CA) to each well. To measure IgA antibodies to ribitol teichoic acid, we added 100 µl of 1:1000 sheep antirabbit IgA, against secretory component and the alpha chain, conjugated with peroxidase (Cooper Biomedical, Malvern, PA) to each well. Both of these antisera gave a single precipitation line when reacted against rabbit serum in immunoelectrophoresis. The plates were incubated for 1 hr at room temperature and then washed with distilled water to remove any unbound antibody. Following this, 100 µl of o-phenylenediamine containing 1% ureaperoxide in 1 M citrate buffer (pH 4.75) was added to each well. The plates were incubated in the dark for 20 min at room temperature. A Titrtek Multiskan (Flow Laboratories, McLean, VA) was used to measure absorbance in each well at a wavelength of 414 nm. Wells containing serially diluted lysozyme or peroxidase-labeled antiserum without rabbit serum, tears or corneal eluate consistently produced absorbance values of less than 0.08. Absorbance readings greater than or equal to 0.1 were considered positive, and ELISA titers were expressed as the reciprocal of the dilution of serum, tears or corneal eluate which gave an absorbance reading of 0.1. Positive and negative controls were run on each plate to ensure consistency of results.

ELISA titers in corneal tissue were calculated using the same approach and formula that have been previously applied to corneal immunoglobulins:

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Ca = \left( \frac{W + Vq}{W} \right) \times Cb
\]

where \(Ca\) is the titer in corneal tissue, \(Cb\) is the titer in the eluting fluid, \(W\) is the weight of tissue in grams, \(V\) is the original volume of eluting fluid in ml and \(q\) is the specific gravity of the cornea. Because the specific gravity of the cornea is approximately 1, weight and volume were considered interconvertible.

Within each immunization group, Wilcoxon's rank sum test on paired data was used to evaluate differences between titers in sera and tears prior to immunization and those at various times after immunization. Corneal titers in the experimental groups at various times after immunization were compared with corneal titers in control rabbits sacrificed at the beginning of the project using the Wilcoxon's rank sum test on unpaired data.

Results

Rabbits immunized with cell wall by the intradermal (Group 1) or topical route (Group 4) and their
controls had normal conjunctiva throughout the experiment. Rabbits immunized by topical application of viable *S. aureus* (Group 3) showed slight conjunctival hyperemia only on days when drops were administered; hyperemia was completely absent when tears were collected three days after drops were discontinued. On the other hand, rabbits immunized by subconjunctival injections of cell wall-complete Freund’s adjuvant (Group 2) and their controls showed conjunctival hyperemia and edema mainly localized to the injection sites in the fornices. When tears were collected, the bulbar conjunctival hyperemia and edema had resolved completely even though dilated vessels were present deep in the fornices at 4 but not 8 and 12 weeks.

The corneas of experimental and control rabbits in all four groups remained clear throughout the 12 week course of the experiment. Light microscopic examination of central and peripheral corneas from all groups at all time points disclosed normal corneas without vessels or inflammatory cells.

Median IgG titers to ribitol teichoic acid in serum, tears and corneas of rabbits before and after intradermal immunization with cell wall (Group 1) are shown in Table 1. Median titers in serum rose over the 12 week course of the experiment. Antibody titers in tears were found at 4 and 8 weeks but disappeared at 12 weeks. Antibody titers were found in the corneas of 14/16 rabbits and reached their highest levels at 12 weeks. Median titers in cornea were higher than those in tears but less than those in serum at all three time points.

Rabbits undergoing subconjunctival immunization with cell wall (Group 2) developed IgG titers to ribitol teichoic acid in serum and tears. IgG titers to ribitol teichoic acid were found in the corneas of all 16 rabbits (Table 2). Unlike rabbits undergoing intradermal immunization in Group 1, these rabbits showed higher median titers of IgG to ribitol teichoic acid in corneas than serum.

After topical immunization with viable *S. aureus* (Group 3), rabbits developed increasing titers of IgG to ribitol teichoic acid in both serum and tears over the 12 week course of the experiment. Titers of IgG to ribitol teichoic acid were found in the corneas of all 16 rabbits (Table 3). At 4 weeks, the median titer in cornea was approximately the same as that in serum, while it was higher than that in serum at 8 but not 12 weeks.

Rabbits in Group 4 undergoing topical immunization with cell wall at two different concentrations developed IgG antibody titers to ribitol teichoic acid in both serum and tears but they were the lowest of all four groups. Only 1/28 experimental rabbits in this group developed a corneal IgG titer of 403 to ribitol teichoic acid.
(Group 1) and two rabbits had titers of 325 and 1201 after topical immunization with viable *S. aureus* (Group 3).

Control rabbits for each experimental group showed no rise in IgG or IgA antibody titers to ribitol teichoic acid in serum or tears throughout the 3 month course of the experiment. Control rabbits had negative IgG and IgA antibody titers to ribitol teichoic acid in their corneas at all time points in the experiment.

**Discussion**

*S. aureus* is the most common cause of serious progressive skin, soft tissue and post-traumatic infections. Exposure to *staphylococcal* cell wall antigens as a result of a tissue infection at a site remote from the eye might correspond to intradermal immunization with these antigens in our study. Intradermal immunization with cell wall (Group 1) resulted in a consistent IgG response to ribitol teichoic acid in serum, tears and corneas and an IgA antibody response in tears but not in corneas or serum. Median titers of IgG to ribitol teichoic acid were highest in serum followed by corneas and then tears. The source of IgG antibodies in the cornea was presumably the serum. After a rise in blood levels, it has been suggested that IgG antibody diffuses into the cornea from the limbal vessels. In this way, IgG antibodies generated in serum in response to a *staphylococcal* infection at a site remote from the eye may diffuse into the cornea where they could play a role in the immunopathogenesis of corneal hypersensitivity responses. Our previous studies have shown that serum antibody levels to ribitol teichoic acid correlated with the development of corneal phlyctenules and catarrhal infiltrates in rabbits after immunization by intradermal or intravenous injections of *staphylococcal* antigens and prolonged topical challenge with viable *S. aureus*.

Subconjunctival immunization with cell wall antigens might correspond to exposure to these antigens in tissues surrounding the eye during a *staphylococcal* infection such as a cellulitis. Exposure to *S. aureus* cell wall in this site resulted in an IgG response to ribitol teichoic acid in serum, tears and corneas and an IgA response to ribitol teichoic acid only in tears but not in serum or corneas. Median IgG titers to ribitol teichoic acid in corneas were higher than those in serum at all three time points. This finding suggests but does not prove that the ocular tissues may be a local source of IgG for the cornea.

Topical immunization with viable *S. aureus* might correspond to a mild conjunctivitis and probably resulted in a more persistent exposure of the eye to *staphylococcal* antigens than topical applications of cell wall which were probably more easily diluted and washed away in the tears. Animals immunized by topical application of *S. aureus* developed IgG titers to ribitol teichoic acid in serum, tears and corneas and IgA titers to ribitol teichoic acid in tears and occasionally in corneas but not in serum. Corneal titers at 4 and 8 weeks suggested that the ocular tissues were an important source of IgG, while corneal titers at 12 weeks might also be influenced by the elevated levels in serum.

The mechanisms responsible for the appearance of IgG antibody in the cornea following ocular immunization with subconjunctival cell wall or topical *S. aureus* are not known. The conjunctival associated lymphoid tissue and/or Langerhans cells may be involved in the recognition and processing of antigen for transport to regional lymph nodes which may then provide T and B lymphocytes to the eye. Plasma cells derived from B cells in the limbal tissue surrounding the cornea may produce antibody that diffuses into the cornea. IgG antibody found in serum after ocular immunization may be another source of corneal antibody. There is a possibility that serum-derived antibodies are preferentially but nonspecifically retained in the cornea. In this study, the tears were probably not an important source of antibody for the cornea because IgG titers in corneas exceeded those in tears in Groups 1–3. Another study which involved the intracorneal injection of antigen demonstrated plaque-forming cells in edematous, infiltrated corneas that could be a source of corneal antibodies. The corneas in our study were clear and histopathologic examination showed no inflammatory cells so that cells within the cornea were probably not an important source of antibody.

IgG titers to ribitol teichoic acid consistently developed in corneas after intradermal and subconjunctival immunization with cell wall and topical immunization with viable *S. aureus*. On the other hand, IgA titers to ribitol teichoic acid were found only occasionally in corneas. These results suggest that IgG responses to *staphylococcal* antigens may be more important than IgA responses in the cornea. The corneal antibodies to ribitol teichoic acid that we demonstrated in this study may play a role in the immunopathogenesis of corneal phlyctenules and catarrhal infiltrates.

**Key words:** *Staphylococcus aureus*, ribitol teichoic acid, corneal antibody, enzyme-linked immunosorbent assay

**References**