Antibody Studies in a Rabbit Model of Corneal Phlyctenulosis and Catarrhal Infiltrates Related to *Staphylococcus aureus*

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Hypersensitivity to ribitol teichoic acid (RTA), the major antigenic determinant of *Staphylococcus aureus*, may be important in a rabbit model of corneal phlyctenules and catarrhal infiltrates. Over a 5-month period, an enzyme-linked immunosorbent assay was used to measure immunoglobulin (Ig) G and IgA antibody levels to RTA in sera, tears, and corneas from rabbits immunized using the following routes: Group 1, intradermal injections of *S. aureus* cell wall (CW) mixed with complete Freund's adjuvant (CFA); Group 2, subconjunctival injections of CW-CFA; Group 3, prolonged topical application of viable *S. aureus* to the eye; Group 4, intradermal injections of CW-CFA plus prolonged topical application of viable *S. aureus*; and Group 5, subconjunctival injections of CW-CFA plus prolonged topical application of viable *S. aureus*. Over the 5-month period, the IgG and IgA antibody levels were correlated to RTA with the development of corneal phlyctenules and catarrhal infiltrates. The IgG titers to RTA were higher than IgA titers in serum, tears, and cornea. The highest antibody titers were IgG titers in cornea. Only rabbits immunized by intradermal or subconjunctival injections of CW-CFA followed by prolonged topical application of viable *S. aureus* (Groups 4 and 5) developed moderate to severe conjunctival hyperemia and edema with corneal phlyctenules and catarrhal infiltrates. When corneal lesions developed between 2-3 months, both groups had the highest corneal IgG and IgA antibody titers to RTA with IgG titers being more than 60 times higher than IgA titers. In the remaining 2 months of the study, the conjunctival response in both groups decreased from moderate-to-severe to mild, and no new corneal lesions developed, despite continued topical application of viable *S. aureus* and elevated antibody titers in cornea, serum, and tears. In this study, IgG and IgA antibody levels to RTA were measured in serum, tears, and cornea in a rabbit model of corneal phlyctenules and catarrhal infiltrates, and the antibody response was correlated with the development of these hypersensitivity lesions. Invest Ophthalmol Vis Sci 32:1854-1863, 1991

We developed a rabbit model of corneal phlyctenulosis and catarrhal infiltrates related to *Staphylococcus aureus*. In this model, rabbits immunized by intradermal and intramuscular injection of phenol-inactivated *S. aureus* followed by topical application of viable *S. aureus* developed vascularized, elevated nodular infiltrates of the cornea, resembling phlyctenules in humans, and peripheral corneal infiltrates running parallel to the limbus and separated from it by a lucid interval, resembling catarrhal infiltrates in humans. These corneal lesions did not have gram-positive cocci in them, suggesting that they represented a hypersensitivity response rather than a direct infection of the cornea. Immunization of rabbits by intradermal injections of *S. aureus* cell wall (CW) mixed with complete Freund's adjuvant (CFA) followed by topical application of viable *S. aureus* also was associated with the development of corneal phlyctenules and catarrhal infiltrates, suggesting that hypersensitivity to CW antigen(s) was responsible for these corneal lesions.

The *S. aureus* CW consists of three major components: peptidoglycan, ribitol teichoic acid (RTA), and protein A. Because immunization with either protein A-CFA or peptidoglycan-CFA was not associated with the development of corneal lesions, we suspected that RTA might be important in their development. Although purified RTA is poorly immunogenic in rabbits even mixed with CFA, rabbits can be immunized to *S. aureus* RTA by coupling it to sheep red blood cells using chromium chloride. When we im-

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Supported in part by National Eye Institute grant EY04606 and the Wasserman Fund.

Submitted for publication: September 7, 1990; accepted January 5, 1991.

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munnized rabbits to RTA by intravenous injections of RTA-sensitized sheep red blood cells, these rabbits developed hemagglutination titers to RTA in sera and corneal phlyctenules after topical application of viable \textit{S. aureus}.\textsuperscript{3} These results suggested that hypersensitivity to RTA, the major antigenic determinant of \textit{S. aureus}, plays a role in the development of corneal phlyctenules and that RTA may represent the antigen that is involved in their immunopathogenesis. Skin tests in these rabbits did not show delayed hypersensitivity to RTA, suggesting that antibody was more important in the development of these corneal lesions.

Because hypersensitivity to RTA may be important in our rabbit model of phlyctenules and catarrhal infiltrates, we measured antibody titers to it using an enzyme-linked immunosorbent assay (ELISA) in sera, tears, and corneas after immunizing rabbits by various routes.\textsuperscript{6} After intradermal immunization with CW-CFA, immunoglobulin (Ig) G titers in the cornea were higher than those in the tears but lower than those in the serum, which was presumably the source of IgG antibodies for the cornea. After subconjunctival immunization with CW-CFA or topical immunization with viable \textit{S. aureus}, IgG titers in the cornea were higher than those in the tears and generally higher than those in the serum, suggesting that the ocular tissues were a local source of IgG. However, IgA titers to RTA were found in tears but not in serum and were found only occasionally in cornea, suggesting that IgG responses to staphylococcal antigens may be more important than IgA responses in the cornea. The results of these studies suggested that corneal antibodies to RTA may be influenced by exposure to staphylococcal antigens not only in the external eye but also at sites remote from the eye.

We measured IgG and IgA antibody levels to RTA in rabbit sera, tears, and corneas after various routes of immunization including such combinations as intradermal or subconjunctival plus topical. Additionally, we followed the antibody response in serum, tears, and cornea over a prolonged period of antigenic stimulation to understand the immunologic response that occurs with chronic exposure to \textit{S. aureus}. Finally, we correlated antibody levels to RTA with the development of corneal phlyctenules and catarrhal infiltrates.

Materials and Methods

\textit{Staphylococcus aureus}

We used a strain of \textit{S. aureus} isolated from a human corneal ulcer. The strain belonged to phage-type 95 and was coagulase-positive, beta-hemolytic, pigmented, and gentamicin sulfate resistant.

Preparation of CW and RTA

The preparation of \textit{S. aureus} CW and RTA followed a modification of methods described previously.\textsuperscript{7} The \textit{S. aureus} was grown in 100 l of tryptic soy broth containing 4 \mu g/ml of gentamicin in a 250-l fermenter (Fermentation Design, New Brunswick, NJ) at 37°C until the maximum logarithmic phase of growth was reached at approximately 6 hr. The cells were harvested and suspended in sterile distilled water. They were disrupted by several passages through a French pressure cell press (American Instrument, Silver Spring, MD) at 20,000 pounds per square inch. The unbroken cells were separated from the broken cells by low-speed centrifugation (2000 rpm for 15 min). The broken cells were removed and centrifuged again at 15,000 rpm for 20 min. The pellet containing the broken cells was treated with 200 \mu g/ml DNase and 200 \mu g/ml RNase (Sigma, St. Louis, MO) to remove nucleic acids and 0.5 mg/ml trypsin (Sigma) to remove protein including protein A. The CW fraction was purified further with 2% sodium dodecyl sulfate (SDS) to remove cell membranes. The SDS was removed by washing with 0.01 M phosphate-buffered saline (PBS), pH 7.6, containing 1 M sodium chloride, and then by washing 11 times with sterile distilled water. The CW was suspended in 70% aqueous phenol and heated at 65°C with constant stirring to remove lipids. The phenol-treated CW was washed 14 times with five volumes of distilled water each time and then lyophilized.

The RTA was extracted from the CW preparation with 10% (w/v) aqueous trichloroacetic acid at 4°C three times overnight on a shaker. The three supernatants were pooled, and the trichloroacetic acid was extracted three times with a double volume of absolute ethyl ether each time. The aqueous phase containing RTA was precipitated with five volumes of 95% ethanol for 72 hr at 4°C. The RTA precipitate was washed three times with acetone, dried, and stored at 20°C in a desiccator.

To evaluate the adequacy of our preparation, CW was fixed in 3% glutaraldehyde in 0.01 M PBS (pH 7.5) and then in 0.1% osmium tetroxide. After dehydration in graded acetone, the CW was embedded in Spurr epoxy resin (Ted Pella, Tustin, CA) and cut. Electron microscopic examination disclosed CW fragments without intact bacterial organisms.

Chemical analysis of our CW (peptidoglycan-RTA) and RTA preparations was done using precolumn derivatization.\textsuperscript{8} The CW contained the following amino acids and amino sugars: N-acetyl muramic acid, N-acetyl glucosamine, glutamic acid, lysine, alanine, glycine, serine, aspartic acid, threonine, valine, isoleucine, leucine, histidine, and arginine. The major com-
ponents of RTA were N-acetyl glucosamine, alanine, and glycine. No N-acetyl muramic acid was detected in the RTA. The chemical composition of our CW and RTA was similar to that reported by others.9,10 Vamcomycin was added to our CW in a final concentration of 0.1% to prevent contamination. The CW (1 mg/ml) was added one drop at a time to CFA (1 mg/ml). A CW emulsion (0.5 mg/ml) was prepared in this way using equal volumes of CW and CFA. The rabbits were immunized with CW rather than RTA because the latter is poorly immunogenic in rabbits even when mixed with CFA. Moreover, immunization routines using RTA-sensitized sheep red blood cells are extremely time consuming and laborious.5

Immunization of Rabbits

We used 156 New Zealand white female outbred rabbits weighing approximately 2 kg: 131 experimental and 25 control rabbits. All investigations described in this manuscript conformed to the ARVO Resolution on the Use of Animals in Research. Before any intradermal or 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 chloral hydrate 42.5 mg/ml and sodium pentobarbital 10.5 mg/ml (Equithesin; UCLA Pharmaceutical, Los Angeles, CA).

Five immunization groups were used. The 27 rabbits in Group 1 were injected in each rear footpad with 0.1 ml of CW-CFA, so that each rabbit received a total of 0.1 mg of antigen. Control rabbits for this group received the same volume of normal saline-CFA injected intradermally into their rear footpads. Two weeks later, the injections were repeated in the front footpads using precisely the same volumes and amounts of CW-CFA or normal saline-CFA.

The 32 rabbits in Group 2 received an injection of 0.1 ml of CW-CFA 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 for this group received the same volume of normal saline-CFA injected intradermally into their rear footpads. Two weeks later, the injections were repeated in the front footpads using precisely the same volumes and amounts of CW-CFA or normal saline-CFA.

The 32 rabbits in Group 2 received an injection of 0.1 ml of CW-CFA 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 for this group received the same volume of normal saline-CFA 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 CW-CFA or normal saline-CFA.

The 32 rabbits in Group 3 were immunized by topical application of a suspension of viable S. aureus in tryptic soy broth (1.5 × 10⁸ colony-forming units/ml). The S. aureus was delivered in tryptic soy broth to enhance the viability of the bacteria and their ability to colonize the external eye as described previously.1 The drops were prepared by growing S. aureus overnight in tryptic soy broth. Each morning, the S. aureus was washed three times in sterile 0.01 M PBS. Finally, the S. aureus pellet was diluted with fresh sterile tryptic soy broth until the optical density reading at 530 nm in a spectrophotometer was 0.19, corresponding to a viable count of 1.5 × 10⁸ colony-forming units/ml. The drops were refrigerated between uses. The rabbits undergoing topical immunization received one drop (approximately 50 μl) in each eye five times daily for 5 days each week for 5 months. In a previous study,1 we showed that this routine maintains S. aureus in the rabbit external eye even on the 2 days each week when they did not receive topical S. aureus. Control rabbits for Group 3 received topical sterile tryptic soy broth, the diluent for viable S. aureus, on the same schedule.

The 20 rabbits in Group 4 received intradermal immunizations 2 weeks apart as described for the rabbits in Group 1. However, 2 weeks after the second intradermal immunization, topical installation of viable S. aureus five times each day for 5 days each week was begun and continued for 4 months. This particular immunization scheme was associated with the development of corneal phlyctenules and catarhal infiltrates in previous studies.2

The 20 rabbits in Group 5 received subconjunctival immunizations 2 weeks apart as described for the rabbits in Group 2. However, topical installation of viable S. aureus was begun 2 weeks after the second subconjunctival injection and continued for 4 months. Control rabbits for Groups 4 and 5 received either intradermal or subconjunctival immunization with normal saline-CFA followed by topical application of tryptic soy broth.

All rabbits were examined weekly for the presence of conjunctival hyperemia and edema (graded as mild, moderate, or severe) and corneal vessels and infiltrates.

Collection of Serum, Tears, and Corneas

In all five experimental and control groups, serum and tears were obtained from all rabbits before immunization. In Groups 1 and 2 undergoing intradermal or subconjunctival immunization, serum and tears were collected from all rabbits 1 month after the first immunizing injection. Additionally, the rabbits were killed from both groups at this time to obtain their corneas. Two, 3, 4, and 5 months after the first immunizing injection, serum and tears were collected from the remaining rabbits, and a minimum of five rabbits was killed from each group at each time to obtain their corneas.

In Group 3 undergoing topical immunization with viable S. aureus, serum and tears were collected from
all rabbits after 1 month of topical immunization. The rabbits were killed at this time to obtain their corneas. After 2, 3, 4, and 5 months of topical treatment, serum and tears were collected from the remaining rabbits, and a minimum of five rabbits was killed at each time to obtain their corneas.

In Groups 4 and 5 undergoing intradermal or subconjunctival injections followed by topical immunization, serum and tears were collected from all rabbits 1 month after the first immunizing injection. The rabbits were not killed at this time to obtain their corneas because Group 1 was equivalent to Group 4 and Group 2 was equivalent to Group 5 at this point. Therefore, corneas for antibody levels were obtained only from Groups 1 and 2. Two, 3, 4, and 5 months after the first immunizing injection, serum and tears were collected from the remaining rabbits in Groups 4 and 5, and five rabbits were killed from each group at each time to obtain their corneas.

Serum and tears were obtained at 0, 1, 2, 3, 4, and 5 months from the control rabbits for each of our five experimental groups, and the rabbits were killed at 5 months to obtain their corneas. Ten normal rabbits were killed at the beginning of the experiment to obtain their corneas for preimmune titers.

Arterial blood was drawn from rabbit ears and centrifuged at 2000 rpm for 15 min to separate the serum from the clot. The tears were collected patiently from both eyes of rabbits by capillary attraction into 5-μl Accupette pipettes (Dade Diagnostics, Aguada, Puerto Rico) as previously described. The tear samples from both eyes were pooled; a total volume of approximately 30 μl was obtained from each rabbit. In Groups 3, 4, and 5, topical application of viable *S. aureus* was discontinued 72 hr before tear collection. Sera and tears were frozen immediately at -70°C.

After death, the central cornea (10 mm in diameter) of both eyes of each rabbit was excised leaving a 1-2-mm rim of peripheral cornea. The excised corneas were rinsed repeatedly with sterile 0.01 M PBS, pH 7.4, and blot-dried to eliminate any possible contribution from tears, blood, or aqueous humor. Occasional sections of clear corneas from each group at each time were placed in 10% neutral-buffered formalin for histopathologic examination. Corneas with infiltrates and vessels were sectioned so that the abnormal corneal tissue was submitted for histopathologic examination and only clear corneal tissue without vessels or infiltrates was used for elutions. The clear corneal tissue from both eyes of each rabbit was minced into small pieces using a #11 scalpel blade, pooled, and added to preweighed plastic test tubes containing 1 ml of 0.01 M PBS, pH 7.4, and eluted at 37°C in a water bath for 72 hr. Then the tubes were centrifuged at 1000 rpm for 15 min, and the supernatant fluid was frozen at -70°C.

**ELISA**

An ELISA was used to measure antibodies to RTA in rabbit sera, tears, and corneal eluates. Purified RTA was dissolved in PBS at pH 7.4 containing 10% carbonate coating buffer (pH 9.6) at a concentration of 10 μg/ml. Each well of flat-bottomed microtiter plates (Nunc Immunoplates; Irvine Scientific, Santa Ana, CA) was coated with 100 μl of RTA. The plates were incubated at room temperature for 18 hr. The plates were washed four times with PBS-Tween 20, pH 7.4, (American Qualex, La Mirada, CA). Eight microliters of serum, tears, or corneal eluate was added to wells in the plates, and serial dilutions were made in PBS from 1:25–1:204,800. The plates were incubated at 37°C for 1 hr and then washed four times with PBS-Tween 20. To measure IgG antibodies to RTA, we added 100 μl of 1:12,000 goat anti-rabbit IgG, heavy and light chain specific, conjugated with peroxidase (American Qualex) to each well. To measure IgA antibodies to RTA, we added 100 μl of 1:6000 sheep anti-rabbit IgA, against secretory component and the alpha chain, conjugated with peroxidase (Cooper, 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 37°C and then washed four times with distilled water to remove any unbound antibody. Then a 100-μl volume of O-phenylenediamine containing 1% urea peroxide 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 Titertek Multiskan (Flow, McLean, VA) was used to measure absorbance in each well at a wavelength of 414 nm. Wells containing serially diluted lysozyme or peroxidase-labeled antisera 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 the results. Antibody titers of selected samples were similar using our purified RTA and that provided to us by Dziarski (Indiana University, Gary, IN). The ELISA titers in corneal tissue were calculated using the same approach and formula used previously to calculate corneal immunoglobulin concentrations and antibody levels:

\[
C_a = \frac{(W + Vq)}{W} \times C_b
\]

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

Statistical Analysis

The ELISA titers in serum, cornea, and tears were converted to logarithms with a base of 2 (log). Statistical analysis was made on the log scale since variance in each immunization group and at each time was stabilized on a log scale, and the distribution of the original titer values was well approximated by a log normal distribution. Mean log titers in serum and tears were compared by repeated-measures analysis of variance. Mean log titers in cornea were compared using a standard two-way (factorial) analysis of variance since the corneas of each rabbit could be obtained only once. For graphic representation of data, mean log titers were converted to median titers in the figures (the antilog of the mean log titer is the median titer). The chi-square test was used to compare the number of corneal lesions in the five groups.

Results

Gross Observations

Rabbits immunized with CW-CFA by the intradermal route (Group 1) and their controls had normal conjunctivas and corneas throughout the 5-month experiment. Those immunized by subconjunctival injections of CW-CFA (Group 2) and their controls showed conjunctival hyperemia and edema mainly localized to the injection sites in the fornices. When tears were collected at 1 month (2 weeks after the second subconjunctival injection), the bulbar conjunctival hyperemia and edema had resolved completely even though dilated vessels were present deep in the fornices at this point but not thereafter. The cornea and bulbar conjunctiva in these rabbits remained normal throughout the experiment.

The rabbits immunized by topical application of viable Staphylococcus aureus (Group 3) showed mild conjunctival hyperemia and edema only on days when the drops were administered; conjunctival hyperemia and edema were absent when tears were collected 3 days after the drops were discontinued. The mild conjunctival hyperemia and edema resolved almost completely at the conclusion of the experiment. Control rabbits for this group that received topical trypsin soy broth were normal throughout the experiment.

The rabbits immunized by intradermal injections of CW-CFA followed by topical application of S. aureus (Group 4) had moderate to severe conjunctival hyperemia and edema during the first 3 months. In this group, 2 of 20 rabbits developed peripheral corneal infiltrates running parallel to the limbus and separated from it by a lucid interval (cataarrhal-like infiltrates) at a mean of 12 weeks after the first immunizing injection (Table 1). Five of 20 rabbits developed vascularized, elevated infiltrates of the cornea 3–4 mm from the limbus (phlyctenules) at a mean of 8.8 weeks after the first immunizing injection (Fig. 1). The conjunctival hyperemia and edema decreased from moderate to severe in the first 3 months to mild at the conclusion of the experiment. Control rabbits for this group were normal throughout the experiment.

The rabbits immunized by subconjunctival injections of CW-CFA followed by topical application of viable S. aureus (Group 5) had moderate to severe bulbar conjunctival hyperemia and edema that developed within 1 week of topical application and persisted for approximately 3 months. One of 20 rabbits in this group developed cataarrhal-like infiltrates 13 weeks after the first immunizing injection. Eight of 20 rabbits developed phlyctenules at a mean of 9.4 weeks after the first immunizing injection. At the conclusion of the experiment, the remaining rabbits showed only mild conjunctival hyperemia and edema. Control rabbits for this group remained normal throughout the experiment.

Table 1. Corneal hypersensitivity lesions in rabbits

<table>
<thead>
<tr>
<th>Immunization groups</th>
<th>No. of rabbits with phlyctenules/total no. of rabbits (%)</th>
<th>Week of development (mean ± SEM)</th>
<th>No. of rabbits with cataarrhal infiltrates/total no. of rabbits (%)</th>
<th>Week of development (mean ± SEM)</th>
<th>Total no. of rabbits with corneal lesions/total no. of rabbits (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (Subconjuctival + topical)</td>
<td>8/20 (40%)*</td>
<td>9.4 ± 1.15</td>
<td>1/20 (5%)</td>
<td>13</td>
<td>9/20 (45%)*</td>
</tr>
<tr>
<td>4 (Intradermal + topical)</td>
<td>5/20 (25%)*</td>
<td>8.8 ± 0.44</td>
<td>2/20 (10%)</td>
<td>12 ± 1</td>
<td>7/20 (35%)*</td>
</tr>
<tr>
<td>3 (Topical)</td>
<td>0/32</td>
<td>—</td>
<td>0/32</td>
<td>—</td>
<td>0/32</td>
</tr>
<tr>
<td>2 (Subconjuctival)</td>
<td>0/32</td>
<td>—</td>
<td>0/32</td>
<td>—</td>
<td>0/32</td>
</tr>
<tr>
<td>1 (Intradermal)</td>
<td>0/27</td>
<td>—</td>
<td>0/27</td>
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* P < 0.01 for difference with Groups 1, 2, and 3.
Antibody Responses in Serum, Tears, and Cornea

The IgG antibody titers to RTA were higher than IgA antibody titers in sera, tears, and corneas. Experimental rabbits in all five groups developed elevated serum IgG antibody titers to RTA over the 5-month course of the experiment (Fig. 2A). Group 4 (intradermal plus topical) and Group 5 (subconjunctival plus topical) had significantly higher titers than the other three groups from months 2–5 (P < 0.05). At month 5, Group 1 (intradermal) had significantly lower titers (P < 0.05), and Group 5 (subconjunctival plus topical) had significantly higher titers (P < 0.05) than the other groups. Groups 2 (subconjunctival), 3 (topical), and 4 (intradermal plus topical) ultimately achieved similar titers.

All five groups developed an IgG antibody response to RTA in their tears (Fig. 2B). The IgG titers in tears were lower in Group 1 (intradermal) than all the other groups from months 2–5 (P < 0.05). At 2 months, Groups 4 (intradermal plus topical) and 5 (subconjunctival plus topical) had significantly higher titers than the other three groups (P < 0.05). At 3 months, Group 5 (subconjunctival plus topical) had significantly higher titers than the other four groups (P < 0.05). The titers were similar in Groups 2 (subconjunctival), 3 (topical), and 4 (intradermal plus topical). As in the case of serum, Group 1 (intradermal) had significantly lower IgG titers (P < 0.05) and Group 5 (subconjunctival plus topical) had significantly higher titers (P < 0.05) than the other groups at 5 months. The other three groups had similar titers at this point.

Normal rabbits killed at the beginning of the experiment had no corneal antibody titers to RTA (Fig. 2C). At 2 and 3 months of the experiment, Groups 4 (intradermal plus topical) and 5 (subconjunctival plus topical) had significantly higher corneal IgG titers than the other three groups (P < 0.05). At 4 months, Group 3 (topical) achieved levels that were similar to Groups 4 (intradermal plus topical) and 5 (subconjunctival plus topical). At 5 months, Groups 3 (topical), 4 (intradermal plus topical), and 5 (subconjunctival plus topical) had significantly higher levels than the other 2 groups (P < 0.05). The cornea developed the highest IgG antibody titers to RTA in this study.

As in our previous studies, there was no IgA response to RTA in serum in any group. All five groups developed an IgA antibody response to RTA in tears (Fig. 2D). Groups 2 (subconjunctival) and 5 (subconjunctival plus topical) achieved the highest titers to RTA that were significantly higher than the other groups—Beginning at month 2 for Group 5 (P < 0.05) and at month 3 for Group 2 (P < 0.05) and continuing throughout the experiment.

At 2 and 3 months, Groups 4 (intradermal plus topical) and 5 (subconjunctival plus topical) had significantly higher titers of IgA antibody to RTA in cornea than the other three groups (P < 0.05) (Fig. 2E). At 5 months, Group 5 (subconjunctival plus topical) was similar to Group 3 (topical), and both were significantly higher than the other three groups (P < 0.05).

Control rabbits for all five groups had no IgG or IgA antibody responses to RTA in sera, tears, or corneas, showing that antibody responses in experimental rabbits were the result of the immunizations and not the result of manipulation and housing in our animal facility.

Histopathologic Findings

Corneal sections corresponding to the sites of the elevated, nodular infiltrates seen on gross observation disclosed nodular elevations of inflammatory cells beneath an intact corneal epithelium (Fig. 3). In some sections, the corneal epithelium over the nodules was thickened, with an increased number of epithelial cell layers. In some sections, the epithelium was invaded by mononuclear cells and neutrophils. Beneath the corneal epithelium, neutrophils and mononuclear cells, including lymphocytes, plasma cells, and macrophages, were scattered throughout the nodules. Vessels surrounded by inflammatory cells were also found in the nodules. Giant cells were not found, and gram-positive cocci were not seen with Gram's stain of corneal sections.

Corneal sections corresponding to the sites of the peripheral infiltrates separated from the limbus by a lucid interval showed anterior stromal accumulations of neutrophils and mononuclear cells, including macrophages, lymphocytes, and plasma cells. Some of the macrophages showed foamy cytoplasm. A few fine
Fig. 2. IgG and IgA antibody responses in serum, tears, and corneas shown on a semilogarithmic plot. The median titers are plotted on the ordinate using a logarithmic scale (base 2), and the months after the first immunization are plotted on the abscissa. Each point represents the median titer ± SEM. Five immunization schemes were used: Group 1, intradermal injections of S. aureus cell wall (CW) mixed with complete Freund’s adjuvant (CFA); Group 2, subconjunctival injections of CW-CFA; Group 3, prolonged topical application of viable S. aureus; Group 4, intradermal injections of CW-CFA plus prolonged topical application of viable S. aureus; and Group 5, subconjunctival injections of CW-CFA plus prolonged topical application of viable S. aureus.

(A) IgG antibody response in serum. Vessels were also found in association with these inflammatory cells. Neither the vessels nor the inflammatory cells extended beyond the peripheral accumulations. The overlying epithelium was intact, without invasion of inflammatory cells. Gram-positive cocci were not seen.

Representative corneal sections from all five groups corresponding to clear corneal tissue that was used for antibody elutions disclosed no vessels or inflammatory cells.

Discussion

The antibody response to S. aureus RTA may be important to study for at least two reasons. First of all, we found that RTA may be the important antigen in our rabbit model of corneal phlyctenulosis. Furthermore, the only serologic tests of clinical importance in the diagnosis of staphylococcal diseases are assays for antibody to RTA, the major antigenic determinant of S. aureus. These assays have been useful in the diagnosis of patients with staphylococcal diseases, including bacteremia with metastatic abscesses, endocarditis with negative blood cultures because of prior therapy, and deep-tissue infections not accessible to culture. In these clinical settings, assays for antibodies to RTA are also useful in assessing response to therapy and relapses.

In the current study, only rabbits immunized by intradermal or subconjunctival injections of CW-CFA followed by topical application of viable S. aureus (Groups 4 and 5) developed moderate to severe conjunctival hyperemia and edema and corneal phlyctenules and catarrhal infiltrates. In previous studies, topical application of CW or inactivated S. aureus to eyes of appropriately immunized rabbits was not associated with the development of conjunctival inflammation and corneal lesions probably be-
cause these applications were a brief, intermittent exposure of the external eye to staphylococcal antigens that were rapidly diluted and drained from the eye in the tears. Viable *S. aureus* organisms were necessary in the conjunctival sac perhaps because they elaborated toxins or other factors that enabled their antigen(s) to gain access to the ocular tissues or perhaps because colonization with *S. aureus* allowed continuous exposure of antigens to the ocular tissues. Topical application of viable *S. aureus* represents not only a form of immunization but also the challenge necessary to elicit corneal lesions. Our routine of applying topical viable *S. aureus* five times each day for 5 days each week maintains *S. aureus* in the rabbit external eye even on the 2 days each week when they did not receive drops. We feel this situation may resemble the human condition where corneal complications develop in patients who have had staphylococcal blepharitis with continuous exposure of the external eye to *S. aureus* for years.

Corneal lesions in Groups 4 and 5 developed only between 2–3 months. The timing for the development of corneal lesions in this study was similar to that in previous studies,1,2 where phlyctenules and catarrhal infiltrates also developed 2–3 months after the first immunizing injection. When corneal lesions developed, Groups 4 and 5 had the highest corneal IgG antibody titers to RTA. Moreover, corneal IgG antibody titers in these two groups were higher than any other titers in this study at 2–3 months. These groups also had the highest IgA titers in cornea at 2–3 months, but IgG titers were more than 60 times higher than IgA titers. Histopathologic examination of representative sections of clear corneal tissue from both groups confirmed that only corneal tissue with-out vessels or infiltrates was used for antibody elutions.

Rabbits in Group 3 undergoing topical application of viable *S. aureus* had corneal IgG titers that eventually approximated those in Groups 4 and 5 at 4–5 months. However, rabbits in Groups 3, 4, and 5 did not develop corneal lesions at 4–5 months. In other words, there appears to be a "window" during which corneal lesions develop and beyond which they do not. This suggests that the immunopathogenesis of these corneal lesions may depend not only on corneal antibody levels but also on the timing of their development.

The reasons for the development of corneal lesions only between 2–3 months, and not beyond this time, are not understood and must be explored. In Groups 4 and 5, the conjunctival response correlated with the corneal response, because conjunctival hyperemia and edema were moderate to severe in the first 3 months of the experiment but only mild at the conclusion of the experiment. It is possible that suppressive mechanisms to the hypersensitivity response develop with time. Additionally, it is possible that progressively increasing antibody titers in tears may more effectively eradicate the organism from the external surface of the eye so that antigen may not be delivered as effectively to the ocular tissues. As opposed to topical immunization with *S. aureus*, intradermal or subconjunctival immunization with CW-CFA may generate different subclasses of IgG that may be important in the development of corneal lesions. Along similar lines, subclasses of IgG or IgA may be generated with the passage of time that have more of a protective rather than hypersensitivity function. Immunologic tolerance to RTA does not appear to be involved because antibody titers tended to increase in serum and tears over the 5-month course of the experiment in
Groups 3, 4, and 5 that had prolonged topical exposure to staphylococcal antigens. Lastly, the IgE antibody response to RTA and its relationship to corneal hypersensitivity lesions should be explored, but skin tests to staphylococcal antigens including RTA never showed immediate reactions in our rabbit model.\(^1,2\)

Our finding that prolonged exposure of the external eye to staphylococcal antigens resulted in a reduction of conjunctival inflammation and no new corneal lesions may have some relevance to human disease. Repeated injections of staphylococcal toxoids and vaccines have been used and recommended as treatment for patients with recalcitrant staphylococcal hypersensitivity diseases of the external eye.\(^3\) However, the mechanism for the beneficial effect of this treatment on ocular hypersensitivity lesions has never been elucidated.

Our results do not prove that the interaction of anti-RTA antibody with RTA in the corneal stroma causes corneal phlyctenules and catarrhal infiltrates. However, a previous study underscored the importance of antibody rather than delayed hypersensitivity to RTA in our rabbit model.\(^3\) Rabbits immunized to RTA by intravenous injections of RTA-sensitized sheep red blood cells developed hemagglutination titers to RTA in serum and corneal phlyctenules after topical application of viable \textit{S. aureus}. Skin tests in these rabbits did not show delayed hypersensitivity to RTA.

The IgG titers to RTA were more than ten times higher in corneas than in serum or tears at 2–3 months. Serum IgG titers to RTA were highest in Groups 4 and 5 and therefore correlated with the development of corneal lesions which were found only in these two groups. The IgG response in tears correlated less well with the development of corneal lesions. The tear IgG response to RTA was highest in Groups 4 and 5 at 2 months but not at 3 months when Group 4 was similar to Groups 2 and 3. It was possible that serum contributed to IgG levels in tears in Groups 4 and 5 with conjunctival and corneal inflammation. However, the source of IgG antibody in tears was not an objective of this study. We were interested in correlating IgG antibody levels in tears, whatever their source, with conjunctival and corneal inflammatory disease.

The IgA titers to RTA were lower than IgG titers in serum, tears, and cornea. As in a previous study,\(^6\) there was no IgA antibody response to RTA in the serum. The highest IgA titers in tears were found in Group 2 (subconjunctival injections) and Group 5 (subconjunctival injections plus topical \textit{S. aureus}). The common denominator of both these groups was the subconjunctival injection which appears to be important in generating IgA antibodies in tears. The IgA titers in tears were not influenced by the serum because there was no IgA antibody response in serum.

The immunization routes that we chose in this study may be relevant to human diseases; \textit{S. aureus} is the most common cause of serious and progressive skin, soft tissue, and post-traumatic infections.\(^3\) Exposure to staphylococcal CW antigens as a result of tissue injection at a site remote from the eye might correspond to intradermal immunization with these antigens in our study. Subconjunctival immunization of CW antigens might correspond to exposure to these antigens in tissues surrounding the eye during a staphylococcal infection such as cellulitis. Topical application of viable \textit{S. aureus} to the eye might correspond to a mild conjunctivitis or to colonization of the conjunctival sac with \textit{S. aureus}. Exposure of tissues to staphylococcal antigens and exposure of the external eye to viable \textit{S. aureus} may be associated with the development of corneal phlyctenules and catarrhal infiltrates in rabbits. It is not known whether a similar mechanism exists for their development in humans.

Little is known about the corneal antibody response to specific antigens. The two groups with corneal lesions had the highest corneal IgG and IgA titers to RTA when the lesions developed. Corneal antibody levels can be influenced by exposure to antigens not only in tissues surrounding the eyes but also in tissues at sites remote from the eye. Tissue exposure to antigen plus topical application of antigen may result in a higher corneal antibody level than either route alone. The highest titers in serum, tears, and cornea were found in Group 5 (subconjunctival plus topical). Our results also demonstrate that, after certain routes of immunization, corneal antibody levels can exceed those in serum or tears. In this study, corneal IgG antibody levels to RTA tended to plateau at a titer of approximately 50,000. To the best of our knowledge, this is the first study to evaluate the corneal antibody response to an antigen that may be involved in the immunopathogenesis of corneal hypersensitivity lesions.

Key words: \textit{Staphylococcus aureus}, phlyctenules, catarrhal infiltrates, antibody, ribitol teichoic acid

References
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