

## Immunization with Alpha-Toxin Toxoid Protects the Cornea against Tissue Damage during Experimental *Staphylococcus aureus* Keratitis

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Received 17 April 2000/Returned for modification 10 July 2000/Accepted 27 July 2000

**Alpha-toxin is a major virulence factor in *Staphylococcus aureus* keratitis. Active or passive immunization with alpha-toxin toxoid could protect against corneal damage. Results show that either form of immunization did not kill bacteria but did significantly protect against corneal pathology, especially epithelial erosion.**

*Staphylococcus aureus* is the leading cause of human corneal infection that may result in loss of visual acuity and blindness (3). Alpha-toxin is produced by approximately 75% of *S. aureus* strains (2, 8, 25) and has been shown to be the major virulence factor in *Staphylococcus* keratitis (6, 21). Considering the importance of alpha-toxin in *S. aureus* keratitis, we examined the effect of passively administered antibody to alpha-toxin and active immunization with alpha-toxin toxoid in a rabbit *Staphylococcus* keratitis model.

*S. aureus* strain 8325-4, an alpha-toxin-producing strain previously analyzed in the rabbit keratitis model (6, 21), was grown to log phase and diluted in tryptic soy broth (Difco Laboratories, Inc., Detroit, Mich.). Alpha-toxin (Sigma, St. Louis, Mo.) purity was determined by the presence of a single band at 33 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20). Toxin was heat inactivated (80°C for 2 h) as confirmed by a loss in hemolytic activity (4, 21).

New Zealand White rabbits (2.0 to 3.0 kg; Myrtle Rabbitry, Thompson Station, Tenn.) were maintained in strict accordance with institutional guidelines and the *Guide for the Care and Use of Laboratory Animals* (13a). Rabbits were anesthetized as described previously (6, 20, 21). For passive immunization, bacteria were mixed (1:1, volume) with either preimmune rabbit sera or rabbit sera containing antibody to alpha-toxin. Each cornea was intrastromally injected with 20  $\mu$ l of the bacteria-antibody mixture containing approximately 100 CFU per cornea (6, 21). Immune sera mixed with tryptic soy broth (1:1, 20  $\mu$ l) were injected into rabbit corneas to determine if the sera induced ocular inflammation. All rabbits were slit lamp examined (SLE) from 10 h postinfection (p.i.) every 5 h until time of sacrifice. SLE of rabbit eyes was performed by two masked observers (6, 20, 21). Corneal erosions were detected using fluorescein, and diameters were measured and expressed in millimeters.

Prior to experimentation, the sera of all rabbits were tested by enzyme-linked immunosorbent assay (ELISA) to ensure the absence of preexisting antibody to *S. aureus* alpha-toxin (5). Specific-pathogen-free rabbits ( $n = 15$ ) were found to lack serum antibody to alpha-toxin. For active immunization, rabbits ( $n = 4$ ) were subcutaneously injected with 50  $\mu$ g of alpha-

toxin toxoid mixed with complete Freund's adjuvant (CFA; Sigma) once a month over a 3-month period. Control rabbits ( $n = 4$ ) were injected with CFA alone. Four weeks following each immunization, sera and tears were collected. Sera were assayed by ELISA for alpha-toxin-specific total antibody or immunoglobulin G (IgG) antibody, using as secondary antibody either anti-rabbit IgG or anti-rabbit gamma heavy chain, respectively. Total antibody and IgA antibody to alpha-toxin in tears were assayed. Sera with IgG titers of 5,000 caused neither agglutination of *S. aureus* nor inhibition of its growth in vitro (data not shown).

Once the serum total antibody titer (as measured by ELISA) to alpha-toxin reached 5,000, the corneas were injected intrastromally with approximately 100 CFU of *S. aureus* 8325-4 (10  $\mu$ l). Rabbits were sacrificed when the SLE score reached 17 or at 45 h p.i.

The number of viable *S. aureus* organisms per cornea was determined by culturing dilutions of corneal homogenates in

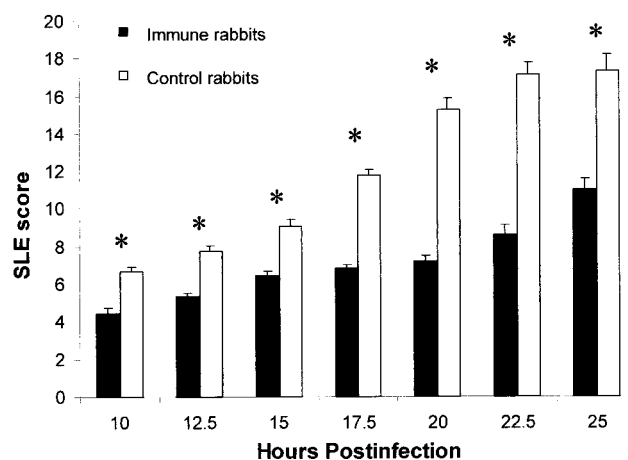


FIG. 1. SLE scores of passively immunized and control rabbits during *Staphylococcus aureus* keratitis. Bacteria were mixed with either immune or preimmune sera at a 1:1 ratio, and the corneas ( $n = 4$ ) were injected with 20  $\mu$ l of the serum-bacteria mixture. A control group consisting of corneas injected only with immune sera was also included. Eyes injected with bacteria and immune sera had significantly lower SLE scores at 20 and 25 h p.i. than eyes injected with bacteria and preimmune sera. Each asterisk denotes a significant difference between groups receiving immune and preimmune sera ( $P \leq 0.0135$ ). Data are expressed as mean SLE  $\pm$  the standard error of the mean (SEM).

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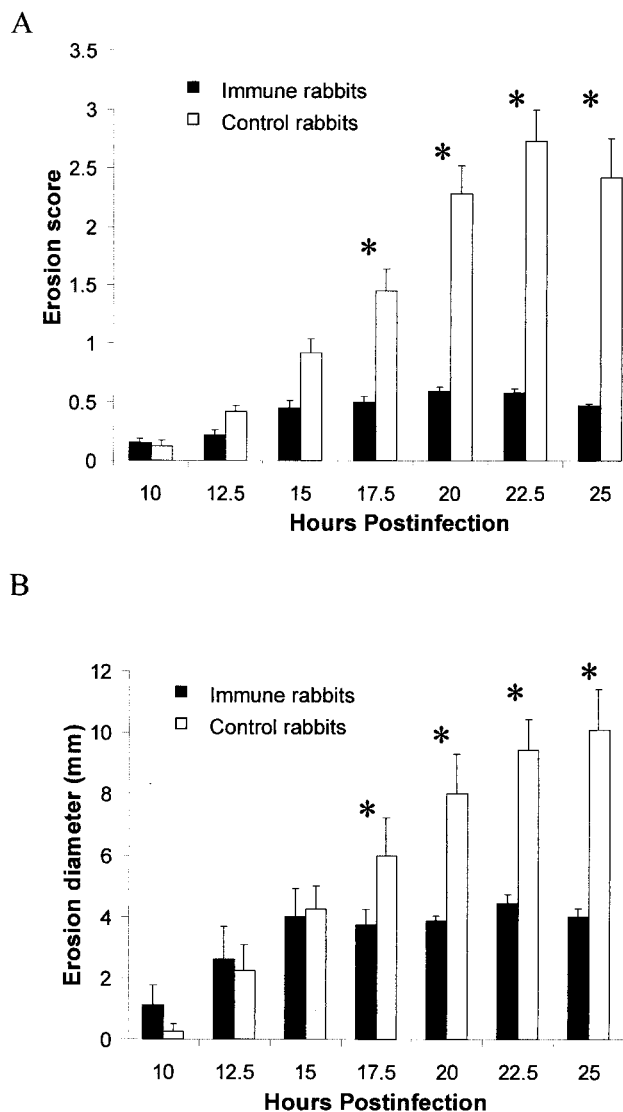


FIG. 2. Development of corneal erosions during *Staphylococcus* keratitis in passively immunized rabbits and control rabbits. Bacteria were mixed with either immune or preimmune sera at a ratio of 1:1, and the corneas ( $n = 4$ ) were injected with 20  $\mu$ l of the serum-bacteria mixture. Erosion score (A) is based on coverage of the cornea and ranked between 0 and 4. Erosion diameters (B) were visualized using fluorescein and measured with a ruler. Both erosion scores and diameters were significantly smaller in passively immunized eyes than in the eyes of control rabbits (treated with preimmune sera) at 20 and 25 h p.i. Each asterisk denotes a significant difference between groups treated with immune or preimmune sera ( $P \leq 0.041$ ). Data are expressed as mean erosion score/diameter  $\pm$  SEM.

triplicate (6, 21). Data were analyzed statistically as described previously (6, 21, 26).  $P$  values of  $\leq 0.05$  were considered significant.

To analyze the protectiveness of passive immunization, serum containing IgG antibody to alpha-toxin (prepared as described above for active immunization) was administered at the time of infection. Passive immunization provided protection to the cornea as evidenced by significantly lower SLE scores for the antibody-treated group than for the control group at 20 and 25 h p.i. ( $P \leq 0.0135$ ) (Fig. 1). Erosions developed at 15 h p.i. in only 25% of antibody-treated eyes whereas 75% of eyes treated with preimmune sera developed erosions. There was

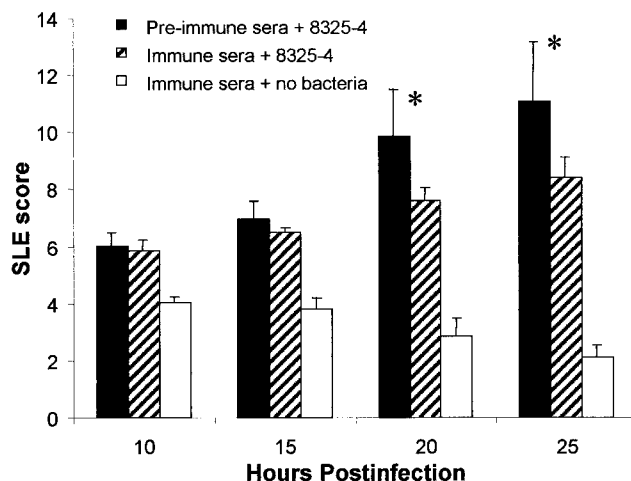


FIG. 3. SLE scores of actively immunized and control rabbits during *Staphylococcus* keratitis. Rabbits were immunized with either alpha-toxin toxoid mixed with CFA (immune) or CFA alone (control) and subsequently infected with approximately 100 CFU of strain 8325-4 ( $n = 8$  per group). Immune rabbits had a significantly lower SLE score than control rabbits at all time points; each asterisk denotes a significant difference between these groups ( $P \leq 0.0004$ ). Data are expressed as mean SLE score  $\pm$  SEM.

no difference in the numbers of bacteria recovered from both groups at 15 h p.i. (immunized =  $5.61 \pm 0.21$  and control =  $5.33 \pm 0.29$  log CFU/cornea;  $P = 0.492$ ). The erosions at 20 and 25 h p.i. were also significantly smaller in antibody-treated eyes than in the eyes of rabbits treated with preimmune sera ( $P \leq 0.041$ ) (Fig. 2).

Active immunization with 2 to 4 injections of toxoid in CFA resulted in serum titers of 5,000 for either total antibody or IgG antibody. However, the IgA titer for alpha-toxin in tears was not substantially increased over that of the preimmune or control rabbits (titers =  $300 \pm 100$ ).

Infection of rabbits actively immunized with alpha-toxin toxoid resulted in significantly less pathology (by SLE score) throughout infection (10 to 25 h p.i.) than infection of control rabbits injected with CFA alone ( $P \leq 0.0004$ ) (Fig. 3). Epithelial erosions were not as extensive in the corneas of immune rabbits compared to those in the corneas of control rabbits from 17.5 h p.i. until time of sacrifice ( $P \leq 0.0001$ ) (Fig. 4). There was no significant difference in the numbers of bacteria (approximately 7 log CFU per cornea) obtained from the corneas of immunized or control rabbits ( $P = 0.3061$ ).

This study has shown, for the first time, that passive or active immunization to alpha-toxin protects the cornea from damage during *Staphylococcus* keratitis. These findings confirm the data from genetic studies (6, 21) and from histopathological studies (20) showing that alpha-toxin is largely responsible for the development of severe tissue damage and inflammation during keratitis. Passive immunization of infected individuals could be useful in limiting tissue damage, particularly in conjunction with antibiotic therapy. Active immunization for *Staphylococcus* infections has been extensively studied (1, 9-12, 15-19, 24) and, for those patients at risk, is a feasible proposition for controlling tissue damage. Vaccination is of increasing priority due to the broadening antibiotic resistance of *Staphylococcus*, including vancomycin resistance (13, 14, 22, 23, 27) and the emergence of methicillin-resistant *S. aureus* infections in the general population (7).

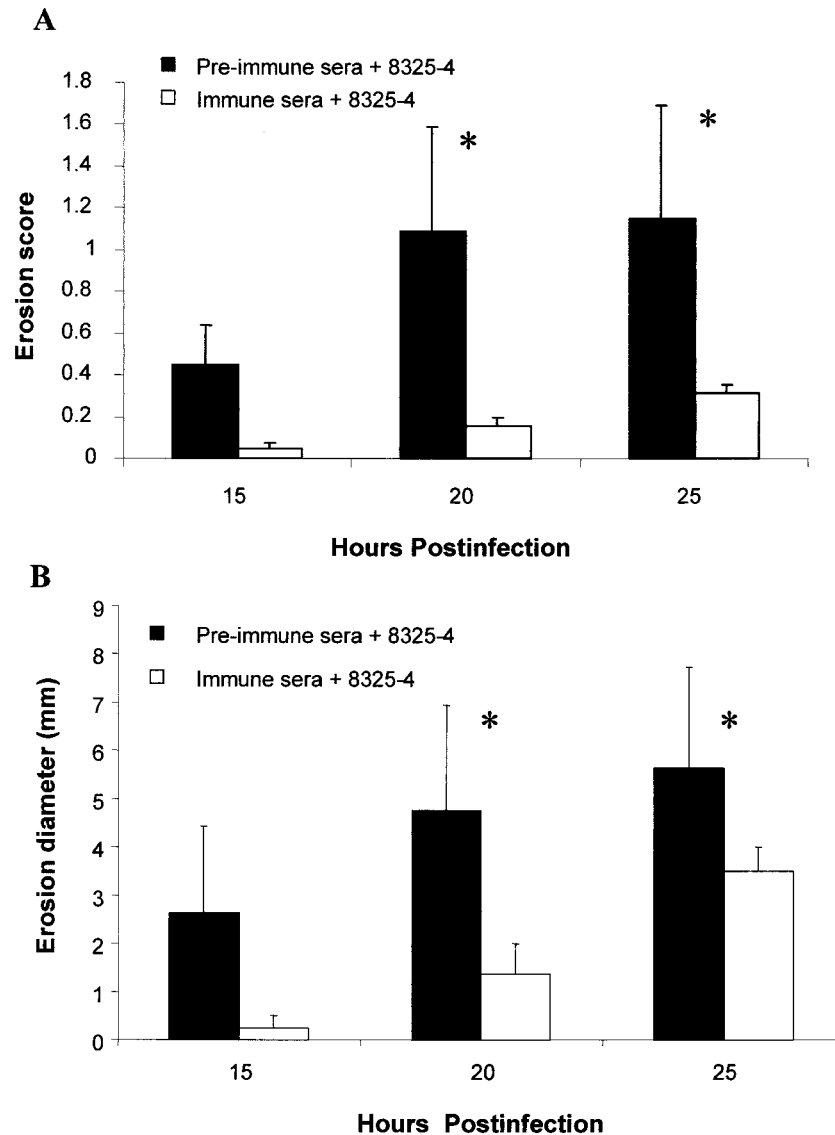


FIG. 4. Development of corneal erosions during *Staphylococcus* keratitis in actively immunized rabbits and control rabbits. Rabbits were immunized with either alpha-toxin toxoid mixed with CFA (immune) or CFA adjuvant alone (control) and subsequently infected with approximately 100 CFU of strain 8325-4 ( $n = 8$  per group). Erosion score (A) is based on coverage of the cornea ranked between 0 and 4. Erosion diameters (B) were visualized using fluorescein and measured with a ruler; values are expressed in millimeters. Erosions (both scores and diameters) in corneas of immune rabbits were significantly smaller than those in control rabbits from 17.5 to 25 h p.i. Each asterisk denotes a significant difference between groups ( $P \leq 0.0001$ ). Data expressed as mean erosion score/diameter  $\pm$  SEM.

We thank Kiana Nelson for her technical assistance.

This research was supported by NIH grant RO1 EY10974.

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Editor: J. T. Barbieri