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NOTES

Passive Immunization with Antiserum to a Nontoxic Alpha-Toxin Mutant from *Staphylococcus aureus* Is Protective in a Murine Model

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A nonhemolytic, nonlethal variant of *Staphylococcus aureus* alpha-toxin constructed via oligonucleotide-directed mutagenesis and containing a single amino acid substitution (H-35 to L) was used to immunize a rabbit. The resulting antiserum was cross-reactive with wild-type alpha-toxin and neutralized its hemolytic activity in vitro. Passive immunization of mice with rabbit antiserum conferred protection against lethal challenge with wild-type alpha-toxin and against acute lethal challenge with a high-alpha-toxin-producing *S. aureus* strain. H35L alpha-toxin may be useful as a protective immunogen in *S. aureus* vaccine studies.

Staphylococcus aureus is an important community-acquired and nosocomial pathogen (17). Morbidity and mortality due to serious disease such as endocarditis, bacteremia, and osteomyelitis are high despite use of antibiotics to which *S. aureus* strains are susceptible. Invasive disease and relapse may occur despite antimicrobial treatment. The growing prevalence of methicillin-resistant and other antibiotic-resistant *S. aureus* strains threatens the effectiveness of current strategies for managing *S. aureus* infection and demonstrates the need for other means of controlling and preventing staphylococcal infections.

Clinical isolates of *S. aureus* produce a variety of extracellular proteins that may play important roles in establishing and maintaining infections (16). One important virulence determinant is alpha-toxin, a pore-forming and hemolytic exoprotein produced by most pathogenic strains of *S. aureus* (7). Alpha-toxin has been shown to be a major virulence factor in certain animal models of infection in which isogenic strains have been used (14, 15). Anti-alpha-toxin immunity has been shown to be protective in neutralizing the detrimental and lethal effects of alpha-toxin in experimental models (1, 4, 12). The relevance of alpha-toxin in human pathophysiology is suggested by the high level of susceptibility of human platelets, endothelial cells, and mononuclear cells to toxin-induced damage (5, 6, 18).

Designing vaccines against bacterial toxins is challenging because of the need to induce a protective immune response without causing illness related to the toxin's biological activity. Inactivation of toxins by chemical means (e.g., the production of tetanus toxoid) has been a useful vaccination strategy. Most previous studies involving immunization of animals with *S. aureus* alpha-toxin have used either a toxoid or the oligomeric, nontoxic form of alpha-toxin that is recovered as a minor constituent during purification (1, 12).

Current molecular biological techniques, including oligonucleotide-directed mutagenesis, can be used to modify the primary structure of toxins and their biological activity. We previously reported a nontoxic alpha-toxin mutant created by replacement of a histidine with leucine at amino acid 35 (H35L toxin) (13). This single substitution rendered the toxin nonhemolytic in a rabbit erythrocyte assay and nonlethal in a murine intraperitoneal model. A liposomal-membrane binding study indicated that the H35L toxin exhibited impaired oligomerization compared with wild-type alpha-toxin. Therefore, histidine 35 appears to be critical for the proper oligomerization necessary for pore formation. In this study, we looked at the immunogenicity of H35L toxin and the cross-reactivity and neutralization properties of anti-H35L serum. We also investigated the ability of passively administered anti-H35L hyperimmune serum to protect mice from lethal challenge with wild-type toxin and a high-alpha-toxin-producing *S. aureus* strain.

Strains used in this study include *S. aureus* DU1090(pDU 1212), from T. J. Foster, Dublin, Ireland (14), and *S. aureus* DU1090(pH35L) (13). DU1090 is a strain in which the alpha-toxin gene (*hla*) has been inactivated by insertional mutagenesis and allelic replacement. DU1090 can be used as a recipient for shuttle vectors containing wild-type *hla* (pDU1212) and inactivated *hla* (pH35L); toxin production is high because of an increased gene dosage effect (10).

H35L toxin was purified from the supernatant of an 18-h tryptic soy broth (Difco, Detroit, Mich.) culture of *S. aureus* DU1090(pH35L) as previously described by using the controlled-pore glass bead chromatography protocol of Cassidy and Harshman (8). Sodium dodecyl sulfate-polyacrylamide (12%) gel electrophoresis of 10 μg of protein revealed a single band of approximately 33 kDa corresponding to the monomeric form of the toxin. The H35L toxin was nonhemolytic at a sensitivity of less than 1 hemolytic unit (HU)/ml in a rabbit erythrocyte assay as described by Bernheimer (2).

Soluble purified H35L toxin was used as an immunogen in a 6-month-old New Zealand White rabbit (Myrtle's Rabbitry, Columbia, Tenn.). A preimmune bleeding was taken before the rabbit was immunized with an emulsion of H35L antigen (1 mg) in 0.1 M phosphate-buffered saline (PBS), pH 7.0, in a 1:1 ratio with complete Freund's adjuvant (Sigma Chemical Co., St. Louis, Mo.). Immunization injections were administered subcutaneously at eight sites (0.25 ml per site) along the back, and 3 weeks later a booster dose of H35L in incomplete

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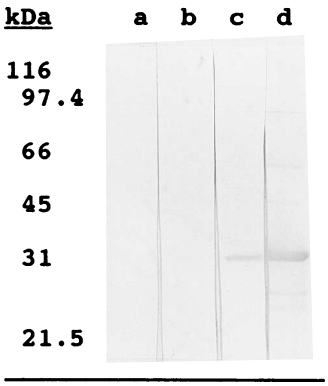

FIG. 1. Immunoblot demonstrating specificity of the antibody response in an immunized rabbit. Purified wild-type toxin (100 ng) (lane a) and crude culture supernatant of DU1090(pDU1212) (30 μg) (lane b) were reacted with preimmune rabbit serum at a 1:500 dilution. Purified wild-type toxin (10 ng) (lane c) and crude culture supernatant of DU1090(pDU1212) (30 μg) (lane d) were reacted with a 1:500 dilution of polyclonal anti-H35L serum.

Freund's adjuvant was given. The rabbit tolerated the immunization series without signs of local inflammation or necrotic reaction. On day 10 following the booster immunization, immune serum was harvested and aliquoted, and it was stored at −20°C until use. Subsequent booster injections were administered at 3- to 4-week intervals, and serum was harvested on day 10 following a booster dose. Serum at each harvest was pooled. All animal studies were approved by the Institutional Animal Care Committee of Vanderbilt University and the Department of Veterans Affairs (Nashville).

Cross-reactivity with wild-type toxin. Pooled immune serum was tested for immunoreactivity to purified wild-type toxin and to crude culture supernatant from *S. aureus* DU1090(pDU 1212) in a Western blot (immunoblot) analysis. Following electrophoresis, two 12% polyacrylamide gels were electroblotted onto a nitrocellulose membrane in Tris–glycine–20% methanol buffer and blocked for 2 h at 25°C with 0.5% Tween 20 in Tris-buffered saline (10 mM Tris, 500 mM NaCl [pH 8.0]) (TSBB). Each membrane was incubated at room temperature either with a 1:500 dilution of preimmune rabbit serum or with anti-H35L immune serum, washed three times in TSBB-Tween, and then incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Boehringer Mannheim, Indianapolis, Ind.). At the end of the second incubation, the membrane was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. Immunization with H35L antigen produced serum that was cross-reactive with wild-type toxin. The strength of the immune response elicited by H35L antigen is demonstrable by the strong immunoreactive 33-kDa band obtained following incubation with a 1:500 dilution of anti-H35L serum against as little as 10 ng of blotted wild-type toxin (Fig. 1). Preimmune serum which was diluted 1:500 did not recognize blotted wild-type alpha-toxin at 10 or 100 ng. The specificity of the polyclonal serum was evaluated by using crude culture supernatant from DU1090(pDU1212) which was adjusted to 30 μg of protein in the immunoblot. Although four faint bands were noted, the strongest immunoreactive band was located at approximately 33 kDa, corresponding to alpha-toxin.

Neutralizing activity of polyclonal anti-H35L serum. To evaluate whether the polyclonal immune serum inhibits the hemolytic activity of alpha-toxin, 0.25-ml serum dilutions of the polyclonal serum in PBS-A buffer (0.145 M NaCl, 0.01 M NaH_2PO_4, 1 mg of bovine serum albumin per ml [pH 7.2]) was incubated with 4 HU of wild-type alpha-toxin for 15 min at room temperature. An equal volume of washed 1% rabbit erythrocytes was then added, and the incubation was continued for 30 min at 37°C. The dilution, or titer, at which no hemolysis was detectable at 545 nm was 1,024. Preimmune serum demonstrated a neutralization titer of 4.

Passive immunization and challenge experiments in mice. (i) Toxin challenge. Two groups of male NIH Swiss mice (20 to 24 g each; Harlan Sprague Dawley, Indianapolis, Ind.) 4 weeks of age were injected in the thigh muscle with 0.2 ml of either preimmune serum or immune serum. The mice were challenged 48 h later with a 0.5-ml intraperitoneal injection of wild-type alpha-toxin at 100 HU diluted in 0.01 M PBS. Death was used as the endpoint, and the time (in minutes) from intraperitoneal injection to death was recorded. Mice that had not died by 24 h were sacrificed by carbon dioxide asphyxiation. The experiment was repeated on another day, and the results were combined. Of the ten mice passively immunized with preimmune serum, nine succumbed to early death (between 2 and 4 h) after challenge with wild-type alpha-toxin. In contrast, none of the 10 mice who had received anti-H35L serum died ($P < 0.001$; Fisher's exact test, two tailed). These results indicate that H35L antiserum effectively neutralizes the lethal activity of alpha-toxin in vivo.

(ii) *S. aureus* challenge. Two additional groups of male NIH Swiss mice 4 weeks of age were passively immunized with preimmune or immune serum. The bacterial inoculum of 2×10^9 CFU/ml was prepared by inoculating 0.5 ml of an overnight culture of *S. aureus* DU1090(pDU1212) into 10 ml of fresh tryptic soy broth and growing to late logarithmic phase for 2.5 to 3 h at 37°C. The bacteria were pelleted at 7,650 × *g*, and the bacterial pellet was resuspended in PBS and washed twice to remove preformed alpha-toxin. Hemolytic activity of the inoculum was negligible (less than 1 HU/ml). Mice were injected intraperitoneally with 10^9 CFU in a volume of 0.5 ml of the adjusted bacterial suspension and were observed over the next 24 h. Five of six mice receiving preimmune serum died between 3 and 4 h after challenge, whereas all six mice receiving immune serum were alive at 24 h after bacterial challenge ($P < 0.02$; Fisher's exact test, two tailed). Survivors were sacrificed at day 10, and the abdominal cavity was aseptically explored. Two of the survivors in the group passively immunized with anti-H35L serum had intra-abdominal abscesses approximately 5 by 3 mm, and four had no evidence of infection. Both abscesses contained purulent material from which *S. aureus* was recovered by culture onto sheep blood agar plates.

Since H35L toxin differs from wild-type alpha-toxin by a single amino acid only, it is not surprising that anti-H35L antibodies demonstrate good cross-reactivity, as is evident by the neutralization of the hemolytic activity of alpha-toxin in vitro and the reactivity in Western blotting. Similarly, Bhakdi et al. recently reported that a His-to-Asn mutation of amino acid 35 yields a nontoxic mutant that is immunogenic in rabbits (3).

Furthermore, we have demonstrated that passive immunization of mice with anti-H35L antiserum prevents death following the injection of wild-type toxin and of a strain which produces large amounts of wild-type toxin but in which preformed toxin had been washed away prior to inoculation. The results show that anti-H35L antibodies can neutralize the lethal effect of alpha-toxin that is given exogenously or produced in vivo. Although all six of the passively immunized mice survived the bacterial challenge, two of the survivors had evidence of intra-abdominal abscess formation 10 days post challenge. This suggests that anti-alpha-toxin antibodies may not fully prevent abscess formation, although the design of this experi-

ment does not allow a more concise inference. The observation would be consistent with the demonstration by Adlam et al. that immunization of rabbits with alpha-toxin toxoid protects against the lethal gangrenous form of *S. aureus* mastitis but does not prevent abscess formation (1).

The great diversity of *S. aureus* as a pathogen and the multitude of virulence factors which it produces make it unlikely that a single immunologic target such as alpha-toxin would be effective as a vaccine candidate. Animal models have shown that anti-alpha-toxin immunity fails to protect against *S. aureus* infection; however, it does appear to reduce infection-associated mortality (9). Clearly, other virulence factors are important in the pathogenesis of *S. aureus* infections. The development of a successful bovine mastitis vaccine composed of a live attenuated *S. aureus* strain attests to the necessity of several staphylococcal components for immunoprotection (19). It is likely that an effective human vaccine, whether subunit-based or live attenuated, will include multiple antigens that elicit antibodies which (i) prevent adherence (e.g., fibronectin-binding protein), (ii) promote phagocytosis (e.g., capsular polysaccharide) (11), and (iii) neutralize toxic effects (e.g., alpha-toxin). A nontoxic alpha-toxin mutant such as H35L may be a useful component of such a vaccine.

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