Neutralization of *Staphylococcus aureus* Exfoliatin by Antibody

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Alum-precipitated exfoliatin was found to be an efficient antigen for eliciting high titers of neutralizing antibody in rabbits. Antitoxin thus produced, and transferred passively, was shown to protect neonatal mice against challenge with two to three lethal doses of preformed exfoliatin even when administration was delayed until 15 min before exfoliation began in control animals. The same dose of antitoxin afforded some protection against six to eight lethal doses of exfoliatin provided it was given before or at the time of challenge. Antitoxin, in the doses given, did not protect against infection with an exfoliatin-producing *Staphylococcus aureus* strain although the onset of exfoliation was delayed.

It has recently been demonstrated that the scalded-skin syndrome in neonatal mice is caused by a protein elaborated by certain *Staphylococcus aureus* strains of phage group II. This protein, called exfoliatin, was partially purified and characterized (2). Subsequently, two other groups (1, 4) succeeded in demonstrating this extracellular toxin and accomplished some degree of purification.

Exfoliatin is rather heat stable, sensitive to trypsin and Pronase, and has a molecular weight of 23,000 as measured by Sephadex chromatography. It is distinct from the alpha and delta toxins also produced by many *S. aureus* strains.

Exfoliatin was shown to be antigenic, and antibody neutralized its ability to cause generalized exfoliation in neonatal mice. The purpose of this study was to explore more fully methods of eliciting neutralizing antibody as well as circumstances dictating its potential usefulness in treatment during the early stages of the disease.

**MATERIALS AND METHODS**

*Staphylococcal strains*. *S. aureus* strain TG (3) was used for production of exfoliatin, and strain EV (3) was used to infect neonatal mice. Inocula were prepared by growing strain EV for 48 hr at 37°C in the same medium used for exfoliatin production (see below). The cells were harvested by centrifugation, washed twice with saline containing 1% (by volume) Trypincase soy broth, and resuspended to the original volume in the same diluent.

**Production of exfoliatin**. Exfoliatin was produced, purified, and assayed as previously described (2). Final preparations contained, per mg, 1,000 to 2,500 50% effective doses (ED<sub>50</sub>) of exfoliatin and 10 to 20 50% hemolytic doses (HD<sub>50</sub>) (2) of alpha toxin. No attempts were made to remove these residual quantities of alpha toxin which constituted 0.1% of the product.

**Rabbit immunization**. Alum-precipitated exfoliatin was prepared by adding 1 ml of 1% aluminum ammonium sulfate to 20 ml of exfoliatin solution (1.5 mg/ml) in distilled water. The mixture was neutralized with 0.1 n NaOH, 2 mg of Merthiolate was added, and the mixture was stored at 4°C. Immunization consisted of a total of 19 mg given as 0.1, 0.3, and 0.6 mg subcutaneously the first week, three intramuscular doses of 0.6 mg the second week, and twelve intravenous injections ranging from 0.3 to 2.0 mg over a month interval.

Exfoliatin was incorporated into incomplete Freund's adjuvant by mixing equal amounts of adjuvant with a saline solution of exfoliatin to give a final concentration of 2 mg/ml. Rabbits were inoculated intradermally with 0.8 mg of exfoliatin in adjuvant and 10 days later with an intradermal injection of 0.8 mg of exfoliatin in saline. This was followed by five additional intramuscular doses (1 to 5 mg) of exfoliatin in saline and then four intradermal inoculations of 1 mg each over a total span of 20 weeks. Each animal received a total of 21 mg of exfoliatin.

**Immunoglobulin concentrate**. Immunoglobulins, for passive immunization studies, were separated from pooled rabbit sera by ammonium sulfate precipitation according to the method of Stelos (6). The final immunoglobulin solution, concentrated four- to tenfold, was stored at -20°C until needed.

**Titration of exfoliatin antitoxin**. To 0.4 ml of serial twofold dilutions of serum was added 0.4 ml of a saline solution of exfoliatin containing 80 ED<sub>50</sub>. The mixtures were allowed to stand at room temperature
for 30 min. A 0.02-ml amount of each mixture was inoculated subcutaneously into at least three neonatal (1 day old) White Swiss mice. Control animals were inoculated with a corresponding dose (2 ED$_{50}$) of exfoliatin in 0.02 ml of saline. Three hours after inoculation, mice were examined for a positive Nikolsky sign or actual exfoliation. The highest dilution of serum which prevented the development of a positive Nikolsky sign in 50% of the mice was considered the anti-exfoliatin titer. Fifty percent end points were determined by the method of Reed and Muench (5).

Titration of anti-alpha toxin antibody. Freshly drawn blood from normal rabbits was mixed with an equal volume of Alsever's solution and stored at 4°C until used. For use, this mixture was centrifuged, the cells were washed twice in saline, and the mixture was suspended in saline to give a 1% suspension.

To 0.4 ml of serial twofold dilutions of serum was added 0.2 ml of alpha toxin (2) in saline (containing 3 to 4 HD$_{50}$). The mixture was allowed to stand for 15 min at room temperature, and 0.4 ml of 1% rabbit erythrocyte suspension was added to each tube. The tubes were incubated for 1 hr at 37°C and centrifuged, the supernatant fluids were quantitatively removed, 4 ml of 0.1% Na$_2$CO$_3$ was added to each supernatant fraction, and the concentration of hemoglobin was determined spectrophotometrically at 430 nm. The highest dilution of serum which reduced hemoglobin release by 50% was the anti-alpha toxin titer.

RESULTS

Exfoliatin antitoxin. Preimmunization sera revealed no anti-exfoliatin or anti-alpha toxin activity. One month after receiving exfoliatin in adjuvant, rabbits had a low but measurable anti-exfoliatin titer, and this was raised to a peak titer of 1:16 with two intramuscular doses of exfoliatin in saline. However, additional injections of exfoliatin in saline failed to maintain these moderate titers (Fig. 1). Immunization with alum-precipitated exfoliatin produced a more rapid response and significantly higher titers.

Although the content of alpha toxin in the antigen preparation was small (0.1%), animals produced appreciable amounts of neutralizing antibody to this contaminant during the course of immunization. With our assay procedure a titer of 1:460 is equivalent to 1 international unit of anti-alpha toxin per ml.

Neutralization of exfoliatin by antitoxin. Ninety neonatal mice were inoculated subcutaneously with 4 ED$_{50}$ of exfoliatin in 0.02 ml of saline. Of these, 14 mice were given 0.02 ml of exfoliatin antitoxin (titer 1:20) subcutaneously 2 hr before exfoliatin. Fourteen mice received the same dose of antitoxin simultaneously with exfoliatin, but at a different subcutaneous site; other groups of 10 to 13 mice each were inoculated with antitoxin 0.5, 1.0, 1.5, and 2 hr after exfoliatin. Fifteen mice, serving as controls, received no antitoxin.

FIG. 3. Protection afforded neonatal mice given exfoliatin antitoxin before and after challenge with 12 ED$_{50}$ of exfoliatin.
Antibody was effective in preventing exfoliation in 75% of neonatal mice when it was given up to 1 hr after challenge with this dose of exfoliatin (Fig. 2). Positive Nikolsky signs were elicited in control mice by 75 min, and antitoxin administered after this time did not protect.

Another group of 103 neonatal mice was given 12 ED50 of exfoliatin subcutaneously. Subgroups received 0.02 ml of antitoxin (titer 1:20) 2 hr before, simultaneous with, and 0.5, 1.0, 1.5, and 2 hr after exfoliatin. These subgroups consisted of 13 to 17 mice each, and 14 mice served as controls. In this case antitoxin protected about 40% of challenged animals and only when administered before or concurrent with exfoliatin inoculation (Fig. 3). Control mice demonstrated positive Nikolsky signs 1 hr after exfoliatin inoculation and were completely exfoliated 2 to 6 hr later.

**Prophylaxis after infection.** Twelve neonatal mice were infected subcutaneously with 10^7 S. aureus strain EV cells in a volume of 0.02 ml. Six mice were inoculated subcutaneously with 0.04 ml of exfoliatin antitoxin (titer 1:20); half of the dose was given 1 hr after infection and the rest 1 hr later. All mice were examined for a positive Nikolsky sign or actual exfoliation 4, 8, 12, and 24 hr after infection. Control animals developed positive Nikolsky signs 8 hr after infection, whereas antitoxin-treated mice did not manifest this response until 12 hr after infection. However, all mice exfoliated and died within 24 hr after infection.

**DISCUSSION**

Among experimental animals, only mice less than 5 days of age are known to be susceptible to exfoliatin. This necessity for neonatal mice imposes difficulties on passive immunization studies by restricting the volume of serum which can be inoculated without encountering excessive leakage from the site. This in turn requires the need for antiserum of high potency. Nevertheless, it was possible, with available antiserum, to demonstrate protection to low doses (4 ED50) of exfoliatin even when exfoliatin antitoxin administration was delayed for 1 hr after challenge. Although antitoxin, in the doses used, delayed the onset of exfoliation in mice infected with S. aureus strain EV, no permanent protection was observed. Presumably this limited amount of passively transferred antitoxin was insufficient to cope with sustained toxin production by the organism.

In humans the scalded-skin syndrome is usually seen during the first 2 years of life. The disease originates as an area of intense erythema usually about the face, neck, or upper trunk. Within a few hours a positive Nikolsky sign can be elicited, and exfoliation begins shortly thereafter. Increasingly larger areas of skin may become denuded during the next 48 hr. Although antibiotics are often employed therapeutically, they appear unable to prevent exfoliation of skin sites originally affected. These studies suggest that with an appropriate hyperimmune serum, of human origin, exfoliation might be prevented or halted if given early during the course of the disease and until antibiotics can eliminate the staphylococci.

Present information indicates that exfoliatin is produced only by S. aureus strains of phage group II. Members of this group comprise about 5 to 10% of staphylococcal isolates. Preliminary studies reveal that approximately 40% of group II strains produce exfoliatin, and only half of these elaborate significant amounts of this toxin. Thus it appears that the risk of infection with an exfoliatin-producing strain during the most susceptible age period is small, and prophylactic immunization during pregnancy or after birth would be impractical. It remains to be seen whether these considerations also preclude the use of pooled human gamma globulin as an effective exfoliatin antitoxin source. One commercial source of staphylococcus antitoxin (Connaught Laboratories) has been examined and found free of exfoliatin-neutralizing activity.

Although an extensive study of various immunizing preparations of exfoliatin has not been done, our findings indicate that the alum-precipitated toxin is an effective antigen and may be of potential value for immunizing selected individuals who could serve as donors for hyperimmune serum.

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**LITERATURE CITED**