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Immunogenicity of *Staphylococcus aureus* Delta-Toxin

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Studies were conducted to determine the immunogenicity of purified *Staphylococcus aureus* delta-toxin. Rabbits and guinea pigs immunized with delta-toxin incorporated into a multiple emulsion consisting of complete Freund adjuvant with 1% Tween 80 produced antibody, whereas animals given toxin in saline or toxin in saline with Tween 80 did not produce antibody. The immunoglobulin G (IgG) fraction isolated by chromatography on protein A-Sepharose was examined for the presence of anti-delta-toxin antibody by immunoelectrophoresis, immunodiffusion, quantitative precipitation tests, affinity chromatography, and toxin neutralization tests. Although delta-toxin-specific IgG precipitated the toxin in agar gels, the antibody did not neutralize the toxin's hemolytic activity. Delta-toxin binding to human erythrocyte membranes was demonstrated by indirect immunofluorescent staining of toxin-treated erythrocytes.

There have been conflicting reports concerning the immunogenicity of *Staphylococcus aureus* delta-toxin. Gladstone and Yosida (7), Hallander (8), and Kantor et al. (10) were unable to produce antibody against purified delta-toxin. More recently, Fackrell and Wiseman (4), Heatley (9), Birkbeck and Whitelaw (1), and Turner (18) reported the production of anti-delta-toxin immunoglobulin in response to immunization of rabbits with milligram quantities of the toxin.

Many investigators have shown that normal sera from a variety of species not only neutralize the hemolytic activity of the toxin, but also form precipitin lines with the toxin in agar gels (3, 7, 9, 10). Whitelaw and Birkbeck (21) have shown that both α and β lipoproteins of normal human serum inhibit the action of delta-toxin. Kapral observed that phospholipids and certain long-chain fatty acids inhibit delta-toxin and suggested that such lipids might be responsible for the neutralizing activity of normal serum (11, 12). Although the presence of nonspecific inhibitors in normal serum must be recognized, they do not pertain to the question of the immunogenicity of delta-toxin.

In this study, immunoglobulin G (IgG) isolated from preimmune and immune sera was examined for the presence of anti-delta-toxin antibody by immunoelectrophoresis, immunodiffusion, quantitative precipitation tests, affinity chromatography, and toxin neutralization tests. Delta-toxin-specific IgG was produced in rabbits and guinea pigs in response to immunization with delta-toxin in complete Freund adjuvant, but the antibodies did not neutralize the toxin's hemolytic activity. It was possible to demonstrate that delta-toxin bound to human erythrocyte membranes by indirect immunofluorescent staining of the toxin-treated erythrocytes.

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MATERIALS AND METHODS

Antigens. Delta-toxin was prepared and purified by using *S. aureus* PG114 as previously described (13). Examination of toxin preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing failed to reveal the presence of contaminants (16). In this study, both the water-soluble and insoluble forms of toxin were used. These had specific activities of 60 and 130 median hemolytic doses per mg, respectively. Bovine serum albumin (BSA), fraction V, fatty acid poor, was obtained from Nutritional Biochemicals Corp.

Antisera. Goat anti-rabbit whole serum, goat anti-rabbit IgG, goat anti-guinea pig whole serum, and goat anti-guinea pig IgG were obtained from Miles Laboratories. A fluorescein isothiocyanate conjugate of the IgG fraction of goat anti-rabbit IgG serum was also obtained from Miles Laboratories.

Animals. Outbred guinea pigs (350 to 800 g) and New Zealand white and Checkered Giant rabbits (2 to 4 kg) were used for immunizations.

Immunizations. Antigens were administered to rabbits and guinea pigs either in a multiple emulsion of complete Freund adjuvant (Difco Laboratories) with 1% Tween 80 (Standard Scientific Supply Corp.), in saline with 1% Tween-80, or in saline alone. Regardless of the vehicle employed, initial inoculations were given to rabbits in 10 simultaneously placed intradermal sites (0.2 mg of antigen in 0.1 ml per site) and to guinea pigs in five such intradermal sites. Animals receiving antigen in Freund adjuvant received additional intramuscular injections every 2 weeks (1 mg of antigen per rabbit; 0.5 mg of antigen per guinea pig). Animals given delta-toxin either in saline or in saline with 1% Tween 80 were boosted eight times at weekly

intervals with 1 mg per rabbit and 0.5 mg per guinea pig administered subcutaneously in the respective vehicles.

Preparation of IgG from serum. IgG was prepared from serum by chromatography on protein A-Sepharose (Pharmacia Fine Chemicals). Serum (2 ml) was passed over a column containing 10 ml of gel. The column was washed with phosphate-buffered saline (PBS) until the effluent reached an absorbance at 280 nm (A_{280}) of <0.01 before being eluted with 0.58% acetic acid in 0.15 M NaCl, pH 2.8 (20). The eluted protein was dialyzed against PBS and concentrated to the original volume of serum by ultrafiltration on an Amicon PM10 membrane. The purity of the IgG fraction was assessed by immunoelectrophoresis. The concentration of pure IgG recovered from sera was estimated by the A_{280}, assuming that the $E^{1\%}_{280}$ (1-cm light path) for guinea pig IgG2 was 13.2 (14) and that for rabbit IgG was 13.8 (17).

Immunodiffusion. Ouchterlony tests were carried out on microscope slides (3 by 1 in. [ca. 7.6 by 2.5 cm]) coated with 4.0 ml of 0.5% Ionagar (Oxoid) in PBS with 0.1% NaN_3. Wells were charged with 5-μl volumes, and the results were read after 40 h of incubation at 23°C.

Immunoelectrophoresis. Electrophoresis of serum and serum fractions was accomplished on slides (3 by 2 in.) coated with 8 ml of gel consisting of 1% agarose in barbital-acetate buffer (pH 8.6, ionic strength = 0.05). A 10-μl amount of 0.05% bromophenol blue was added to each 100 μl of serum as a marker dye. The wells (2-mm inside diameter) were charged with 10 μl of serum and electrophoresed at a constant current of 10 mA per slide. Troughs were cut and charged with 40 μl of the appropriate antiserum or antigen. After developing for 40 h at 23°C, the slides were washed and dried, and the agar film was stained with either Coomassie brilliant blue R-250 or Oil Red O (CI 26125).

Screening of animals. To eliminate animals which were spontaneously exposed to staphylococcal products, preimmunization sera were examined for the presence of neutralizing activity for *S. aureus* alpha-toxin. Alpha-toxin was prepared and purified with *S. aureus* P-78 as previously described (13).

Serial twofold dilutions of sera were prepared in PBS. To 0.2-ml portions of each serum dilution was added 0.1 ml of alpha-toxin solution containing four median hemolytic doses. After mixing and standing in an ice bath, 0.2 ml of a 1% (vol/vol) chilled rabbit erythrocyte suspension was added, and the tubes were held at 0°C for 15 min before incubation at 37°C for 1 h. After centrifugation, the supernatants were visually compared with supernatants from unlysed controls to determine the greatest serum dilution that neutralized the toxin. Each test was run in duplicate.

Neutralization of delta-toxin. Whole sera and purified IgG fractions were tested for their ability to neutralize the hemolytic activity of delta-toxin. Serial twofold dilutions of samples were made in PBS with a final volume of 0.2 ml. To each dilution was added four median hemolytic doses of delta-toxin in 0.1 ml. A 0.2-ml amount of a 1% (vol/vol) human erythrocyte suspension was added to each tube, and the tubes were incubated at 37°C for 20 min. After centrifugation, the supernatants were compared visually, and the highest dilution of serum that gave no hemolysis was considered to be the neutralization endpoint. Each test was run in duplicate.

Immune precipitation curve. Portions (1 mg) of rabbit IgG obtained from preimmune or immune serum were added to various amounts of delta-toxin in a final volume of 1.2 ml. The tubes were incubated for 48 h at 4°C and centrifuged at 1,500 × *g*. The precipitate was washed twice with ice-cold PBS, and the washed precipitate was dissolved in 0.8 ml of 0.1 N NaOH. The protein content of the precipitate was estimated by A_{280}.

Coupling of delta-toxin to Sepharose. The method recommended by Pharmacia Fine Chemicals was followed. A 3-g amount of dry CNBr-activated Sepharose 4B was washed and reswollen on a sintered-glass filter with 1 mM HCl. A 100-mg amount of soluble delta-toxin dissolved in the coupling buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3) was added to the gel, and the resultant slurry was mixed end over end for 2 h at 23°C. Excess toxin was removed by washing the gel with coupling buffer, and any remaining reactive groups were blocked by suspending the gel in 1 M glycine-HCl buffer, pH 8.0. The gel was washed on a sintered-glass filter alternating four times between coupling buffer and 0.01 M acetate buffer, pH 4.0. Although at this stage the A_{280} of the washings was <0.005, it became apparent in subsequent experiments that additional delta-toxin could be eluted from the gel with 1.0 M acetic acid. This was thought to be the result of a high degree of toxin-toxin aggregation due to hydrophobic bonding. To deaggregate and elute the noncovalently bound toxin, the gel was washed with a graded series of ethanol solutions in water (25 to 100%). The resultant gel was found to have coupled 8.5 mg of delta-toxin per ml of gel, as determined by subtraction of the amount eluted (determined by the A_{280}). A control gel (glycyl-Sepharose) was prepared as described above, except that no toxin was added, and all of the reactive groups were blocked with glycine.

Affinity chromatography. Columns containing 2.0 ml of either glycyl- or delta-toxin-coupled Sepharose were washed just before application of IgG with several bed volumes of tris(hydroxymethyl)aminomethane (Tris)-buffered saline, pH 8.0, followed by an equal volume of 1.0 M acetic acid. Columns were reequilibrated with Tris-buffered saline, and the IgG was applied to the gel in the same buffer. Unbound protein was removed with Tris-buffered saline, and the bound IgG eluted with 1.0 M acetic acid.

Indirect immunofluorescent staining of delta-toxin-treated erythrocyte membranes. In these studies, freshly drawn human blood from a single donor was used. The erythrocytes were washed and stored in PBS at 4°C. Erythrocyte ghosts were aseptically prepared by the method of Dodge et al. (2) and used as either 4 or 10% (vol/vol) suspensions. Intact erythrocytes were diluted in PBS to make a 4% (vol/vol) suspension. Thin blood smears were also prepared from the same donor, air dried, and fixed by immersion in absolute methanol for 30 s.

Erythrocytes and ghosts were stained in suspension. A 0.5-ml amount of the erythrocyte or ghost suspen-

sion was pipetted into glass centrifuge tubes and held in an ice bath. To the chilled suspension was added either 1, 10, or 100 μg of soluble delta-toxin, and the final volume was adjusted to 1.0 ml. The tubes were warmed at 37°C for 20 min and then centrifuged at 10,000 × *g* for 20 min in the cold. The supernatants were discarded, and the pellets were washed twice with 2.0-ml portions of ice-cold PBS. To the washed pellets was added 0.1 ml of a 1:5 dilution of immune rabbit IgG. The rabbit IgG was previously absorbed with packed erythrocytes to remove any nonspecific agglutinins possibly present in the preparation. The tubes were held at 0°C for 30 min, after which time the pellets were suspended in 2.0 ml of PBS, and the unbound IgG was removed by washing the pellet twice more with similar amounts of PBS. The fluorescein isothiocyanate conjugate (0.1 ml of a 1:32 dilution) was added to the pellet, and the tubes were held at 0°C for 30 min. The pellet was again washed twice with 2.0-ml portions of PBS and resuspended to the same volume. Suspensions were examined as wet mounts by using an ultraviolet light microscope.

Thin blood smears were stained with the same reagents described above. Two drops of a 1-, 10-, or 100-μg/ml solution of delta-toxin was applied to a ringed area of fixed smears, and the slides were held at room temperature for 15 min. Any unbound toxin was removed by washing the slides with PBS. The primary rabbit antibody was applied to the same area on the slide and allowed to react for 15 min. The unreacted IgG was washed away with PBS, and the smear was similarly stained with the fluorescein isothiocyanate conjugate. The slides were again washed with PBS, and the stained area was covered with 1 drop of glycerol and a cover glass. At no time during the staining procedure were the smears allowed to dry.

RESULTS

Immunizations. Delta-toxin did not form a stable emulsion when mixed with complete Freund adjuvant even after sonication. This was presumed to be due to interaction of the toxin with Arlacel A (mannide monoleate), thus rendering it ineffective as an emulsifying agent. The problem was circumvented by adding 1% Tween 80 to the mixture to form a multiple emulsion. This emulsion remained stable for at least 24 h at room temperature.

Delta-toxin-specific IgG was found in the sera of rabbits and guinea pigs within 4 weeks after initial exposure to the antigen in adjuvant. Animals given delta-toxin in saline (either soluble or insoluble forms of the toxin) or in saline with 1% Tween 80 (soluble form) did not produce detectable antibody after eight weekly injections (total antigen per rabbit, 8 mg; total antigen per guinea pig, 4 mg).

Animals given BSA in adjuvant (as a multiple emulsion) produced sera with precipitation titers as high as 1:64 in the rabbits and 1:8 in the guinea pigs.

Preparation of IgG from sera. IgG prepared from either rabbit or guinea pig serum was free of other serum components as judged by immunoelectrophoresis. Recoveries were as high as 15 mg of IgG per ml of rabbit immune serum and 8 mg of IgG per ml of serum from immune guinea pigs.

Immunodiffusion. Nonimmune sera from rabbits and guinea pigs were found to precipitate delta-toxin in agar gel double diffusion. The lines of precipitation thus formed stained with Oil Red O, suggesting that the material interacting with delta-toxin was lipoprotein. IgG isolated from nonimmune sera did not precipitate delta-toxin in agar gels. IgG from immune sera however, was able to precipitate the toxin, and the precipitate stained with Coomassie brilliant blue but not with Oil Red O.

IgG from early immune sera gave a single line of precipitation with delta-toxin, whereas IgG from some later antisera (10 weeks) gave a double line of precipitation. To evaluate whether both lines contained delta-toxin, attempts were made to dissolve the precipitate or interfere with its formation by use of Tween 80. With IgG preparations that gave two lines of precipitation, only the line forming closest to the antigen well was subsequently solubilized by Tween 80 (Fig. 1). However, by incorporating 1% Tween 80 into the agar before diffusing delta-toxin against anti-delta-IgG, the formation of both lines of precipitation was prevented. The line of precipitation resulting from the interaction of BSA and anti-BSA serum was neither dissolved nor prevented by Tween 80.

Agar gel diffusion done in gels containing 2% (vol/vol) washed human erythrocytes showed that the lines of precipitation were located well within the zone of hemolysis and that the zone of hemolysis was not truncated (Fig. 2).

The precipitation titers obtained with rabbit and guinea pig immune IgG recovered after 10 weeks of immunization are shown in Table 1.

Immunoelectrophoretic analyses. Rabbit and guinea pig preimmune sera, when electrophoresed before being interacted with delta-toxin, did not show precipitation in the IgG region of the gel (Fig. 3). However, sera from both nonimmune and immune animals consistently showed a line of precipitation which developed in the prealbumin region. This precipitation line stained with Oil Red O (Fig. 4). Sera from either immunized rabbits or guinea pigs exhibited, in addition, a line of precipitation in the IgG region of the gel (Fig. 3).

Sera from animals immunized with BSA showed no precipitation of delta-toxin in the IgG region, indicating that nonspecific precipitation

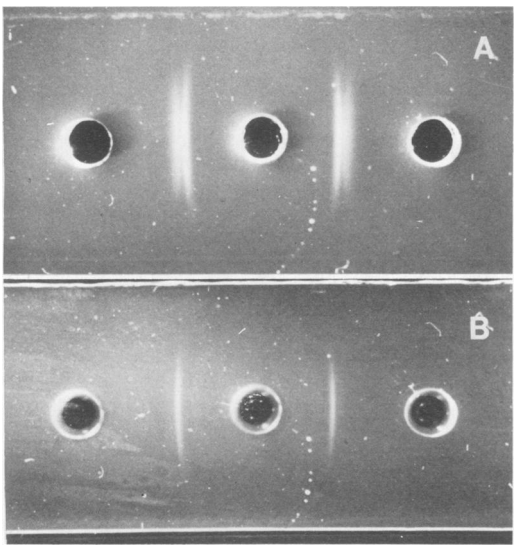

FIG. 1. *Solubilization of precipitation lines with Tween 80. A. The center well contained 5 μl of immune rabbit IgG, and the outer wells contained 5 μl of delta-toxin (1 mg/ml). B. Same as (A) above, but 24 h after both troughs were filled with 10% Tween 80. Lines closest to antigen wells were dissolved.*

of the toxin by the IgG fraction did not occur (Fig. 3).

Toxin neutralization. Sera from all animals used in this study were examined for anti-alpha-toxin antibody before initiation of the immunization schedule. No animal had detectable neutralizing activity for alpha-toxin (titer of <1:2.5), suggesting that these animals had no prior contact with *S. aureus* through natural exposure.

Preimmune and immune whole sera from both rabbits and guinea pigs were able to neutralize the hemolytic activity of delta-toxin, but no significant difference in titers was found between sera obtained before or after immunization. The IgG fraction from preimmune sera had no delta-toxin neutralizing activity. Likewise, IgG prepared from immune sera was unable to neutralize delta-toxin even though it could precipitate the toxin (Table 2).

Immune precipitation curve. Immune rabbit IgG (1 mg per tube) gave a typical immune precipitation curve when reacted with various amounts of delta-toxin (Fig. 5). The region of maximum precipitate formation occurred with approximately 60 μg of toxin. Preimmune IgG did not precipitate delta-toxin at any concentration tested. Although attempts were made to demonstrate the precipitation of delta-toxin by assaying residual toxin in the supernatants, this was unsuccessful because the hemolytic assay was not sensitive enough to measure the small concentrations of toxin present (<20 μg/ml).

Affinity chromatography. Delta-toxin coupled to Sepharose was used as a solid-phase immunoadsorbent to isolate delta-toxin-specific IgG molecules (Fig. 6). Comparable IgG fractions passed through glycyl-Sepharose columns served to control nonspecific adsorption and to assess the percent recovery of applied immunoglobulin. Equal amounts of preimmune or immune IgG (3.3 mg) were applied to 2.0-ml portions of each gel. Delta-toxin-Sepharose was able to bind some of the IgG from immune sera but none of the IgG fraction from preimmune sera. Based upon the A_{280}, the total IgG recoveries from both gels ranged from 94 to 101%. Approximately 15% of the immune IgG applied was shown to be specific for delta-toxin.

This delta-toxin-specific IgG could be eluted with 1.0 M acetic acid, but not with 0.1 M

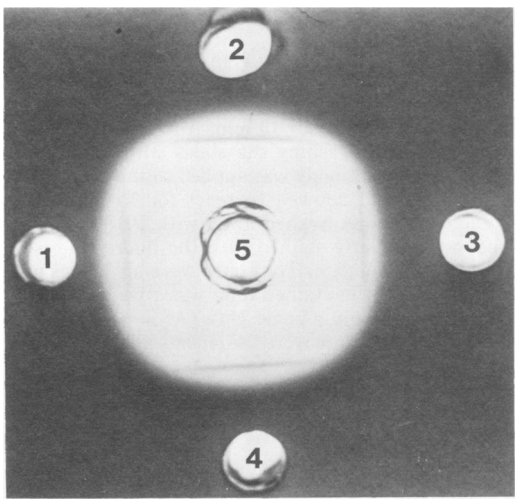

FIG. 2. *Immunodiffusion in agar with 2% human erythrocytes. Wells 1 to 4 contained 5 μl of rabbit immune IgG. Well 5 contained 5 μl of delta-toxin (1 mg/ml). The slide was incubated for 18 h at 4°C and then warmed to 37°C for 2 h to permit lysis.*

TABLE 1. *Delta-toxin precipitation titers of immune IgG from sera 10 weeks into the immunization schedule*

Animal[a]	Precipitation titer[b]
R1	1:4
R2	1:8
R3	1:8
R4	1:8
GP1	1:2
GP2	1:4
GP3	1:4

[a] R, Rabbit, GP, guinea pig.

[b] Determined in 0.5% Ionagar by using 2-mm (inside diameter) wells in a hexagonal array against soluble delta-toxin (1 mg/ml). Results were read after 40 h at 23°C.

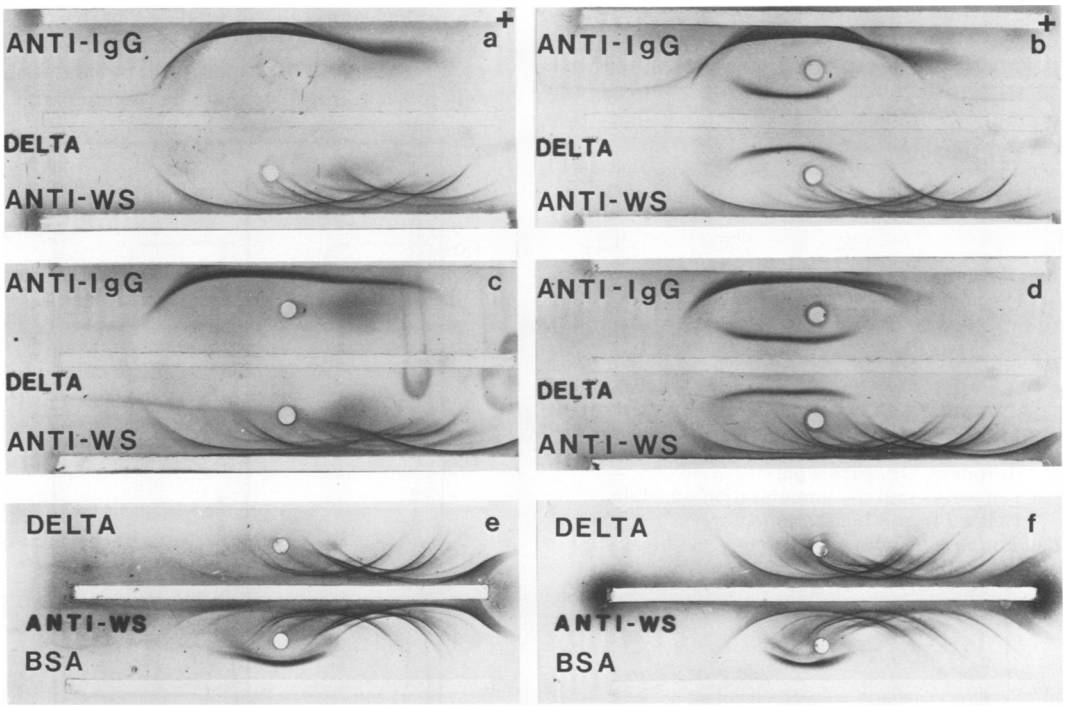

FIG. 3. *Immunoelectrophoresis of rabbit and guinea pig sera. Wells contained 10 μl of rabbit preimmune serum (a), rabbit anti-delta-toxin serum (b), guinea pig preimmune serum (c), guinea pig anti-delta-toxin serum (d), guinea pig anti-BSA serum (e), or rabbit anti-BSA serum (f). Troughs contained 40 μl of the appropriate goat anti-IgG, goat anti-whole serum (WS), delta toxin (1 mg/ml), or BSA (1 mg/ml). The anodes are to the right.*

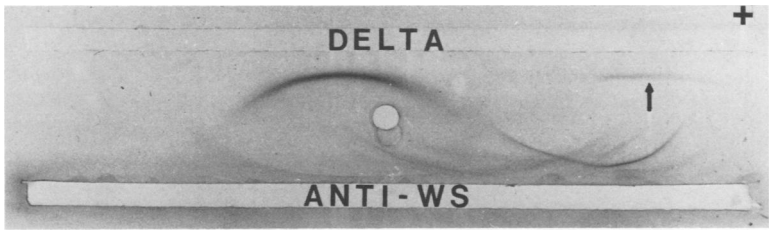

FIG. 4. *Oil Red O staining (arrow) of nonspecific precipitation band. The center well contained 10 μl of rabbit immune serum, and the troughs contained 40 μl of either delta-toxin (1 mg/ml) or goat anti-rabbit whole serum (WS). Dried agar films were stained sequentially in Oil Red O and Coomassie brilliant blue R-250.*

glycine-hydrochloride buffer, pH 2.5. Protein eluted with acetic acid was subsequently confirmed as being anti-delta-toxin IgG by its precipitation after immunodiffusion against goat anti-rabbit IgG and by its ability to precipitate delta-toxin. The IgG which did not bind to the immunoadsorbent failed to form a line of precipitation when diffused against the toxin.

In another experiment, 7.4 mg of immune rabbit IgG was loaded onto 2.0 ml of delta-toxin–Sepharose and was resolved into two fractions, one eluting with 0.1 M acetic acid, and the other eluting with 1.0 M acetic acid (Fig. 7). Both fractions were able to precipitate the toxin agar gels.

Indirect immunofluorescence. Treatment of intact erythrocytes with either 1, 10, or 100 μg of delta-toxin per ml followed by indirect immunofluorescent staining revealed the presence of large amorphous masses of intensely fluorescent material and an occasional uniformly fluorescent ghost. When the same field was observed alternately with either ultraviolet or visible light, intact erythrocytes were never seen to fluoresce (Fig. 8). Immunofluorescent staining of delta-toxin–treated erythrocyte ghosts resulted

TABLE 2. *Delta-toxin neutralization titers[a] of preimmune and immune whole sera and immune IgG*

Animal[b]	Titer		
	Whole serum		Immune IgG[d]
	Preimmune	Immune[c]	
R1	1:20	1:20	<1:2.5
R2	1:10	1:10	<1:2.5
R3	1:10	1:10	<1:2.5
R4	1:5	1:10	<1:2.5
GP1	1:5	1:5	<1:2.5
GP2	1:5	<1:5	<1:2.5
GP3	1:5	<1:5	<1:2.5

[a] The test toxin dose was four times the median hemolytic dose; each test was run in duplicate.
[b] R, Rabbit; GP, guinea pig.
[c] Ten weeks into the immunization schedule.
[d] Isolated from whole serum 10 weeks into the immunization schedule.

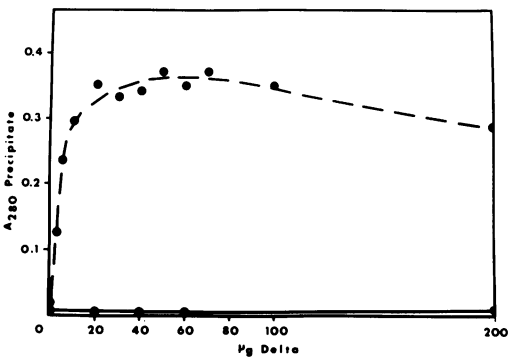

FIG. 5. *Immune precipitation curve. Tubes contained various amounts of delta-toxin and 1.0 mg of either preimmune (solid line) or immune IgG (dashed line) from the same rabbit.*

in suspensions in which virtually every ghost fluoresced. Regardless of the delta-toxin concentration used, all ghosts exhibited fluorescence, but the intensity of fluorescence increased with increasing concentrations of toxin. With suspensions of ghosts, the large amorphous fluorescent masses were not observed (Fig. 8).

When erythrocytes were first treated with delta-toxin and then hypotonically lysed, the resultant ghosts upon staining were found to fluoresce. This suggested that the intact erythrocytes must have had toxin associated with their membranes. The erythrocytes in methanol-fixed thin blood smears, when reacted with delta-toxin and stained, were also found to stain uniformly. In this case, the delta-toxin treatment described above (100 μg/ml) did not result in lysis of the fixed cells.

No fluorescent staining was seen with erythrocytes or ghosts not treated with delta-toxin nor with toxin-treated preparations reacted with the fluorescein isothiocyanate conjugate alone.

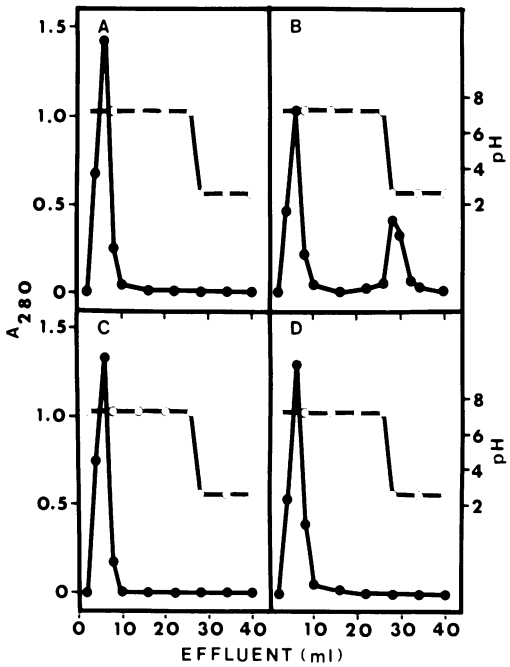

FIG. 6. *Affinity chromatography of preimmune and immune rabbit IgG. Immune IgG (3.3 mg) was chromatographed on either 2.0 ml of glycyl-Sepharose (A) or delta-toxin–Sepharose (B). Preimmune IgG (3.3 mg) was chromatographed on either 2.0 ml of either glycyl-Sepharose (C) or delta-toxin–Sepharose (D). Samples were applied in Tris-buffered saline and eluted with 1.0 M acetic acid. Symbols: ●, A_{280}; ○, pH.*

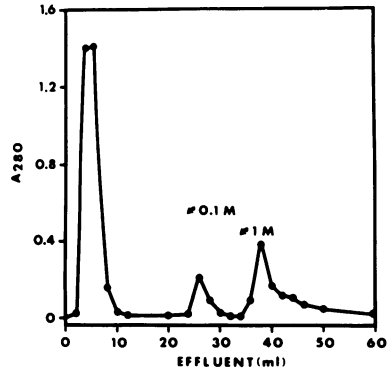

FIG. 7. *Affinity chromatography of 7.4 mg of immune rabbit IgG applied to 2.0 ml of delta-toxin–Sepharose in Tris-buffered saline. Unbound IgG was washed through with Tris-buffered saline. Arrows indicate beginning of elution with 0.1 M and 1.0 M acetic acid.*

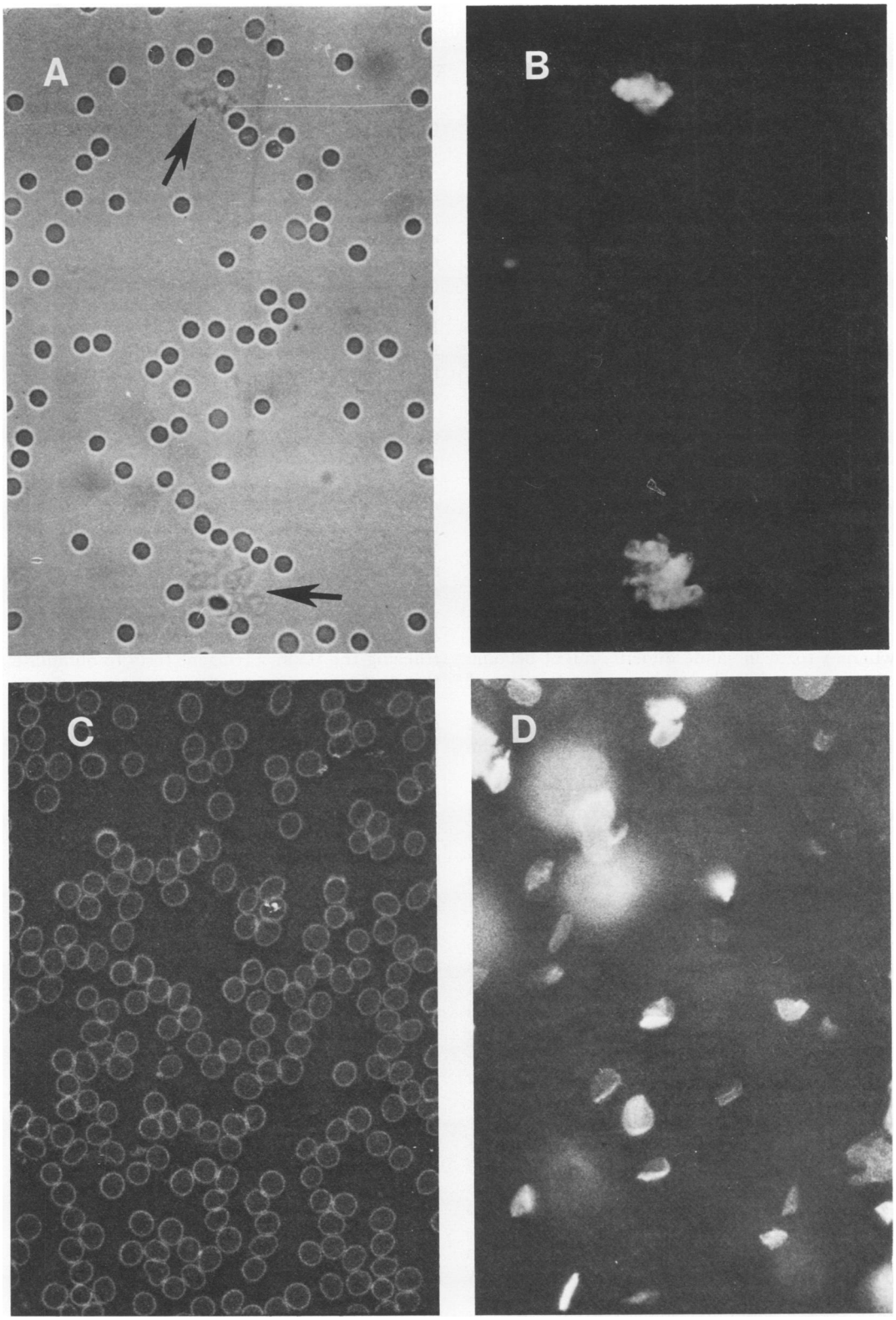

FIG. 8. *Indirect immunofluorescent staining of delta-toxin–treated human erythrocytes. A. Erythrocytes (2% [vol/vol]) were treated with 10 μg of delta-toxin per ml and stained in suspension. The field was illuminated with visible light. Arrows indicate amorphous masses. ×250. B. Same field as above illuminated with ultraviolet light. C. Methanol-fixed thin blood smear treated with 100 μg of delta-toxin per ml and stained. ×250. D. Erythrocyte ghosts (5% [vol/vol]) were treated with 100 μg of delta-toxin per ml and stained in suspension. ×400.*

DISCUSSION

Since animals can be exposed to *S. aureus* through natural means, we elected to examine the sera from all subjects for evidence of preexisting antibody to the alpha-toxin since this protein is a potent immunogen. All animals used in these studies were found to be free of anti-alpha-toxin antibody and, therefore, were presumed to have had no prior contact with the organism.

Immunization of rabbits and guinea pigs with soluble delta-toxin in complete Freund adjuvant containing 1% Tween 80 resulted in the production of delta-toxin-specific IgG. The relatively high precipitating titers of the immune IgG preparations indicated that the animals responded well to immunization with this preparation. Animals immunized with delta-toxin in saline or with toxin in saline containing 1% Tween 80 failed to produce anti-delta-toxin antibody. Since complete Freund adjuvant with 1% Tween 80 forms a multiple emulsion, the slow release of the antigen could explain its efficacy. It is also possible that the interaction of toxin with Arlacel A would permit better uptake of the antigen, whereas toxin in saline diluents might become associated with cell membranes at the site of injection, and, in the absence of an adjuvant-stimulated cellular infiltrate, the immune system might never become effectively exposed to the toxin.

The question of the immunogenicity of delta-toxin can only be answered by comparison of the immunoglobulin fractions from nonimmune and immune sera. IgG was prepared from whole sera by chromatography on protein A-Sepharose and was shown to be free of other serum components by immunoelectrophoresis. This method was chosen because it allowed us to process rapidly a large number of serum samples while providing good recovery of IgG with a high degree of purity. Furthermore, protein A binds both IgG1 and IgG2 subclasses of guinea pig immunoglobulin (6), and guinea pig IgG1 is difficult to recover free of contamination with other serum proteins by ion-exchange chromatography (14).

Nonimmune sera from a variety of species precipitate delta-toxin in agar gels and neutralize its hemolytic activity. These effects have been considered by some investigators to be due to the antibody (15), whereas other investigators view them as reflecting an interaction of toxin with lipoproteins of phospholipids (3, 10, 12). By immunoelectrophoretic analyses, it was possible to examine preimmune whole sera, without prior fractionation, for the presence of delta-toxin-reactive IgG. No precipitation occurred in the IgG region, but a precipitation line did develop in the prealbumin region. Since the precipitation line stained with Oil Red O, it seemed likely that the serum component interacting with delta-toxin was a lipoprotein. Other evidence that antibody was not responsible for precipitation or neutralization by normal sera was the inability of nonimmune IgG to react with delta-toxin after electrophoresis, immunodiffusion, quantitative precipitation, affinity chromatography on delta-toxin–Sepharose, or in toxin neutralization tests. In addition, IgG fractions from rabbits hyperimmunized with BSA had no delta-toxin specificity as demonstrated by immunoelectrophoresis.

Our findings differ in certain aspects from those of other workers. Turner and Pickard (19) reported delta-toxin neutralizing activity associated with IgG fractions prepared by ammonium sulfate precipitation of nonimmune and immune sera. Heatley (9) and Birkbeck and Whitelaw (1) also reported delta-toxin neutralization by IgG fractions prepared by ion-exchange chromatography. It is questionable whether these methods would result in IgG free of contaminating lipoproteins capable of neutralizing the toxin. Stringent tests to eliminate this possibility were not done.

Fackrell and Wiseman (4) showed a coincidence of the edge of hemolysis with the precipitation lines and a truncation of the zone of hemolysis when delta-toxin was diffused against anti-delta-toxin IgG (purified by ion-exchange chromatography) in agarose containing 1% human erythrocytes. We found no such coincidence or truncation of the hemolytic zone as a result of immunodiffusion of delta-toxin in agar with 2% human erythrocytes against immune rabbit IgG. Fackrell and Wiseman did not report whether their anti-delta-toxin preparation had detectable neutralizing activity, but it is possible that their immunoglobulin preparation was contaminated with lipoproteins.

We found no detectable delta-toxin neutralizing ability associated with IgG isolated with immune sera. This cannot be explained simply as a poor antibody response since high precipitating titers were obtained with the same IgG fractions. The lack of toxin neutralization, even at IgG concentrations that gave significant precipitation, might be due to combination of the antibody with sites on the toxin molecule not involved in interaction with the cell membrane, such that the resultant immune complexes would remain hemolytically active. Another explanation would be that the antibody is directed toward a nonhemolytic contaminant. However, the delta-toxin used in this study was shown to be free of protein contaminants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

and isoelectric focusing (16). Furthermore, results of immunofluorescence studies indicate that immune IgG can still combine with delta-toxin already attached to erythrocyte membranes. This finding tends to substantiate the explanation that the antigenic determinant and membrane combining site are distinct and spatially separate.

We have noticed that incorporation of 1% Tween 80 into the agar gel completely inhibited precipitation of delta-toxin by anti-delta-toxin IgG but did not affect the precipitation of BSA by anti-BSA serum. This finding may be explained if the toxin, in its monomeric form, is a univalent antigen. Molecular weights ranging from 68,000 to 200,000 have been reported for delta-toxin. Kantor et al. (10) found by sucrose density-gradient centrifugation a delta-toxin subunit with a molecular weight of 21,000 in the presence of 0.1% Tween 80 and a molecular weight of <10,000 when the Tween 80 concentration was increased to 1.0%. Gel filtration of delta-toxin in 6 M guanidine-hydrochloride yielded an estimated molecular weight of the polypeptide chain of 5,000. Recently, Fitton et al. (5) found the molecular weight of the toxin monomer to be 2,977, thus lending credence to the possibility that the toxin is a univalent antigen. If this were the case, then only aggregated forms of toxin would precipitate with antibody. This could explain why in agar gels containing erythrocytes the zone of hemolysis extended beyond the line of precipitation with antibody (Fig. 2). Antibody complexed with toxin monomers would remain soluble and would be able to diffuse past the precipitated toxin aggregates, thus causing lysis of erythrocytes. Further experiments measuring the binding capacity of immune IgG in the presence of Tween 80 might confirm this hypothesis.

Results from immunofluorescent staining of toxin-treated erythrocytes indicated that delta-toxin can be demonstrated in association with membranes by use of a specific immunological probe. This would imply that either the toxin does not penetrate so deeply into the lipid bilayer that the antigenic determinant(s) on the molecule is unavailable to interact with the primary antibody or that toxin associates with the membrane in a polymeric form. The difference in fluorescence seen when erythrocytes and ghosts were stained in suspension is difficult to interpret. Initially, it was thought that hemoglobin in intact cells might quench fluorescence, but the explanation cannot be that simple since methanol-fixed unlysed erythrocytes, when treated with toxin and stained, did fluoresce. The large amorphous fluorescent masses seen when intact erythrocytes were toxin treated and stained appeared to be composed of clumped membranes from toxin-lysed cells. The clumping of such delta-toxin-induced ghosts could be mediated either by the primary antibody or the secondary antibody. However, if this were the case, one would expect similar clumping of stained ghosts prepared by hypotonic lysis, and this was not observed.

The failure to demonstrate fluorescence with unlysed erythrocytes stained in suspension and the occurrence of fluorescence when delta-toxin-treated erythrocytes were subsequently hypotonically lysed might be due to the presence of a substance loosely associated with the erythrocyte outer membrane which could mask the bound toxin. This material might be removed during the lysis and washing procedure with PBS. If this substance were also destroyed or removed by methanol, it could explain why unlysed erythrocytes stained in fixed smears fluoresce.

Our findings suggest that delta-toxin is immunogenic, but the antibody does not interfere with its ability to interact with cell membranes. Therefore IgG specific for delta-toxin should prove to be a useful immunological reagent for determining the localization of toxin in tissues and, consequently, might assist in determining what role, if any, delta-toxin plays in the host-parasite interaction.

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