Failure to Detect Cell-Associated Enterotoxin B in *Staphylococcus aureus* by Immunofluorescence

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Enterotoxin B-producing and -nonproducing *Staphylococcus aureus* strains showed cell fluorescence when tested with fluoresceinisothiocyanate-labeled rabbit anti-enterotoxin B globulin, probably as a result of a protein A-immunoglobulin G (Ig G) interaction. No cell-bound enterotoxin B could be detected by immunofluorescence using F(ab')₂-fragments of anti-enterotoxin B globulin. However, soluble enterotoxin B could be estimated by immunofluorescence. Approximately 1,000-fold more enterotoxin B was detected by immunodiffusion as an extracellular product in the media than could be detected in the cell fraction. The results show that intact Ig G is not suitable for the detection of antigens other than protein A on the cell surface of *S. aureus* in conventional immunofluorescence. For such purposes, the use of F(ab')₂-fragments of Ig G is recommended.

Results indicating that staphylococcal enterotoxin B is associated with the cell surface have been presented. Enterotoxin formation can be inhibited by Tween 80, oleic acid, sodium deoxycholate, penicillin, D-cycloserine, or bacitracin (15). L-forms of enterotoxin B-producing *Staphylococcus aureus* strains do not produce enterotoxin B (7). Fluorescent antibody studies by Friedman and White (16), Stark and Middaugh (31), and Morse and Baldwin (26) strongly suggested that enterotoxin B is associated with the cell surface.

*S. aureus* is a well known cause of nonspecific reactions with nonimmune- and immune sera in the immunofluorescence technique (2, 4–6, 23–25). These nonspecific reactions have been a major problem, and many methods have been introduced to eliminate them (2, 4, 8, 9, 12, 22). Genigeorgis and Sadler (17, 18) found nonspecific cell fluorescence of *S. aureus* when using fluorescein-conjugated anti-enterotoxin B and had difficulties in studying cell-bound enterotoxin B. They based their method of detecting enterotoxin B in culture media and food on the demonstration of specific fluorescent precipitates around the bacterial cells and not on the presence of fluorescent cells alone.

In a previous report from this laboratory (12), the interaction between protein A on the cell surface of *S. aureus* and the Fc-part of immunoglobulin G (Ig G) was shown to give rise to nonspecific immunofluorescence reactions. With fluoresceinisothiocyanate (FITC)-labeled F(ab')₂-fragments, obtained by pepsin digestion of Ig G, it was shown that it is possible to eliminate the nonspecific reaction completely while retaining specific activity of the F(ab')₂-fragments. The present work was undertaken to investigate the possible role of protein A in cell-associated immunofluorescence reactions using enterotoxin B antiserum. Our data suggest that enterotoxin B is not detectable on the cell surface.

**MATERIALS AND METHODS**

**Organisms.** *S. aureus* types Cowan I, S6, and Wood 46 were used. In addition, 10 *S. aureus* strains producing enterotoxin B and 10 strains not producing enterotoxin B from our own collection were used. *Neisseria gonorrhoeae* was isolated in the routine laboratory.

**Production of enterotoxin B.** A semisynthetic medium (3) with 2% protein hydrolysate (1) and 1.5% agar (Difco) was used as a solid medium for the production of extracellular enterotoxin B. The staphylococci were cultured for 20 hr at 37 C on the medium which was covered with cellophane (19). The cells were harvested with 10 ml of phosphate-buffered saline solution and were centrifuged for 15 min at 4,500 rev/min.
Purification of enterotoxin B. The purification of enterotoxin B (20) involved three steps. In step one, the enterotoxin was concentrated in dialysis tubing in polyethylene glycol, dialyzed against 0.02 M sodium phosphate buffer (pH 7.2) containing 0.1 M NaCl, and was gel filtered on a column of Sephadex G 100 at 1.5 to 2 ml per cm² per hr. Enterotoxin was recovered in a second peak. In the second step, fractions with high enterotoxin activity were pooled, concentrated, dialyzed, and further purified by preparative electrophoresis in 0.05 M barbital buffer (pH 8.15) on a Sephadex G-25 column (50 by 2.2 cm). The electrophoresis was run toward the cathode at 500 V for 24 hr and then eluted in 2.5-ml fractions. In step three, after concentration, the enterotoxin B peak from step two was further purified by preparative electrophoresis in 0.045 M sodium phosphate buffer, pH 5.95. The electrophoresis was performed as described above.

Production of specific antisera. For vaccine production, the purified enterotoxin B fractions were dialyzed for 24 hr at 4°C against 0.02 M sodium phosphate buffer (pH 7.2) containing 0.1 M NaCl. Formaldehyde (35%) was then added to a concentration of 0.2% (v/v). After incubation for 24 hr at 37°C, the material was dialyzed against the same buffer for another 24 hr. Equal volumes of vaccine and Freund's complete adjuvant (Difco) were mixed with a Turmix device in the cold. A 0.5-ml fraction was injected into each hindleg of a rabbit. The procedure was repeated after 14 days and then each month for 4 months. Fourteen days after the last injection, the animal was bled and killed. The anti-enterotoxin thus obtained was adsorbed with heat-killed S. aureus, strain Wood 46, to remove antibodies against antigens other than enterotoxin B. The serum was further tested against enterotoxin B with reference to a specific anti-enterotoxin B serum kindly supplied by M. S. Bergdoll, University of Wisconsin. Antiserum to N. gonorrhoeae was raised according to current methods at the Institute of Medical Microbiology (8).

Preparation of Ig G. Rabbit Ig G was prepared from sera by precipitation three times at a 37% saturation with ammonium sulfate. After dialysis against 0.0175 M Na₂HPO₄ (pH 6.3), the globulin fraction was chromatographed on a diethylaminoethyl (DEAE) cellulose column equilibrated and eluted with the dialysis buffer (13). The first peak to emerge from the column was concentrated in an ultrafiltration cell (Diaflo M 50, Amicon Corp.) to a protein concentration of 18 to 20 mg/ml and dialyzed against 0.15 M NaCl. The Ig G preparation gave a single Ig G line in immunoelectrophoresis against antirabbit plasma protein serum (Behringwerke AG).

Pepsin digestion. To obtain Fab(½)₅ fragments of Ig G, pepsin digestion was carried out by the method of Nisonoff (28). Pepsin (Worthington Biochemical Corp.) was used in the proportion 1 mg of enzyme per 50 mg of protein. To serve as a control, purified anti-N. gonorrhoeae Ig G was added before digestion to the mixture of pepsin and purified anti-enterotoxin B Ig G.

Conjugation. Conjugation with FITC was performed by the method of Nairn (27). FITC (0.05 mg) was added per mg of Ig G or Fab(½)₅-fragments of Ig G. Before conjugation, the concentration of Ig G was adjusted to 10 mg/ml, and that of Fab(½)₅-fragments of Ig G was adjusted to 6.6 mg/ml to give equivalent concentrations on a molar basis. Molar extinction coefficients of 1.36 and 1.48 at 280 nm for Ig G and Fab(½)₅-fragments, respectively, were used. After conjugation, nonreactive FITC was removed by passing the solution through a Sephadex G-25 column (27).

Staining of smears and recording of reactions. Smears were prepared from 20-hr cultures on cellophane-covered Cassman agar plates or blood-agar plates. A platinum loop was lightly dipped into the culture, and its contents were smeared into one drop of phosphate-buffered saline (PBS), pH 7.2, on a slide. Control tests were performed with N. gonorrhoeae grown on GC medium (Difco).

Heat fixation of the smears by rapidly passing the slides through a gas flame six times, fixation in acetone for 2 min, fixation in cold ethanol for 1 to 10 min, fixation in 10% Formalin for 1 to 10 min, and no fixation at all were tried. Then, one drop of conjugate was placed on the smear, and the slide was incubated for 20 min at 37°C. After being washed with PBS, the smear was examined under a fluorescence microscope with the following standards of cell fluorescence: 3+, intensely fluorescent margin, well marked edges; 2+, faintly fluorescent margin, edges usually diffuse; 1+, barely distinguishable fluorescent margin with diffuse edges. Fluorescent precipitates were detected as described by Genigeorgis and Sadler (17).

Staining of living cells. The membrane filter (Millipore Corp.) method of Genigeorgis and Sadler (17) was used.

Immunoelectrophoresis. Immunoelectrophoresis was performed by the method of Scheidegger (29) using Veronal buffer at pH 8.6.

Disintegration of S. aureus cells. S. aureus S6 was cultivated in 2,000 ml of nutrient broth. Aeration was achieved by shaking on a platform shaker. After being washed once with PBS, the bacteria were suspended in 40 ml of PBS. The suspended bacteria (15 ml) were disintegrated nine times in an x-press by the method of Edebo (10). After disintegration, the solution was centrifuged to remove cell walls. The supernatant fluid was tested by immunodiffusion against anti-enterotoxin B serum.

Lysostaphin digestion. S. aureus S6 cultivated on cellophane-covered Cassman medium (12) was harvested and washed twice with saline and once with distilled water. A suspension (5 ml) of 10⁶ bacteria per ml in 1.2 M sucrose, 0.02 M tris(hydroxy)methylaminomethane (Tris)-acetate buffer, (pH 8.0) was incubated at 37°C with 0.5 mg of lysostaphin (Mann Research Laboratories). At intervals, 0.05-ml samples were pipetted into tubes containing 3 ml of 0.02 M Tris buffer. After 20 min of incubation, the optical density of the sample had fallen to 10% of the original, and the digestion was stopped by centrifugation at 5,000 x g for 20 min. The supernatant
fluid containing digested cell walls was dialyzed against 0.02 M Tris buffer. After dialysis, the supernatant fluid was concentrated 10 times in dialysis tubing on polyethylene glycol (Kebo AB) and was tested by immunodiffusion against antienterotoxin B serum.

**Determination of protein A.** Protein A was extracted from *S. aureus* by the method of Jensen (21). The extract was diluted approximately 1:1 in 0.1 M Tris-hydrochloride (pH 8.0) so that 1 ml of the final solution of crude protein A corresponded to 5 ml of the broth culture of *S. aureus* containing 10^6 colony-forming units per ml. Protein A was quantitated by means of the hemagglutination technique (30) with doubling dilutions of the material to be assayed.

**Immunodiffusion.** Titration of enterotoxin B in gel was performed against antienterotoxin B serum in 1% agar (special Noble agar, Difco) in 0.15 M NaCl.

**RESULTS**

**Characterization of anti-enterotoxin B serum, Ig G and F(ab')_2-fragments.** The anti-enterotoxin B serum obtained by immunization of rabbits with purified formaldehyde-treated enterotoxin B and adsorbed with *S. aureus*, strain Wood 46, was characterized by immunoelectrophoresis against crude toxin and purified formaldehyde-treated toxin (Fig. 1). The slight difference in electrophoretic mobility is explained by the formaldehyde treatment of the purified toxin. The figure shows that the antisera was highly specific. Immunodiffusion in agar gel of the adsorbed serum against purified enterotoxin B showed an antibody titer of 1/160, which is of the same order as or higher than that for a reference serum supplied by M. S. Bergdoll. The reference serum from Bergdoll and our preparation gave a reaction of identity when tested against purified formaldehyde-treated enterotoxin B.

**F(ab')_2-fragments obtained by pepsin digestion of rabbit antiantenterotoxin B Ig G were conjugated with FITC by using standard methods (27). The conjugate obtained was tested in immunodiffusion against purified enterotoxin B. Precipitates were obtained with the same dilutions as for intact Ig G.**

**Reactions with anti-enterotoxin B Ig G and F(ab')_2-fragments in immunofluorescence.** Table 1 is a summary of typical experiments performed with anti-enterotoxin B Ig G and its F(ab')_2-fragments to differentiate between nonspecific reactions with protein A and specific reactions with cell-associated enterotoxin B in immunofluorescence. *S. aureus*, strain S6, which has a high production of enterotoxin B as determined by gel diffusion and has a significant content of protein A as shown by the hemagglutination titer, gave an immunofluorescence reaction with intact Ig G but not with F(ab')_2-fragments of anti-enterotoxin B Ig G. *S. aureus*, strain Cowan I, with no production of enterotoxin B, but with a high content of protein A, gave a strong immunofluorescence reaction with anti-enterotoxin B Ig G, but no reaction with F(ab')_2-fragments of the globulin. *S. aureus*, strain 4916, which produces enterotoxin B, but not protein A, gave no immunofluorescence reaction with the Ig G or F(ab')_2-fragments of the globulin.

*S. aureus*, strain Wood 46, producing neither enterotoxin B nor protein A, was negative in all tests shown in the table. In the anti-enterotoxin B Ig G preparations used, anti-*N. gonorrhoeae* Ig G was included as a control. In that system, FITC-labeled Ig G and F(ab')_2-fragments of the immunoglobulin gave the same titer when tested against *N. gonorrhoeae*.

*S. aureus* strains with a moderate to high production of enterotoxin B as determined by gel diffusion against anti-enterotoxin B serum (titer 1/10 to 1/160) gave bright pericellular precipitates as described by Genigeorgis and Sadler (17) in immunofluorescence with FITC-labeled anti-enterotoxin B. The reactions recorded for FITC-labeled anti-enterotoxin B Ig G and F(ab')_2-fragments of the globulin were similar. This fluorescent pericellular precipitate is apparently due to a specific reaction between soluble enterotoxin B and anti-enterotoxin B Ig G or F(ab')_2-fragments of the globulin.

In an attempt to exclude the possibility that the enterotoxin B was destroyed or lost from the cell surface during preparation of the smears, five different conventional methods for fixation of the slides were tried with similar
results. Enterotoxin B-producing strains did not show any cell fluorescence with anti-enterotoxin B F(ab\(^1\))\(_2\)-fragments. Living cells of *S. aureus*, strain S6, were stained with FITC-labeled anti-enterotoxin B Ig G and F(ab\(^1\))\(_2\) fragments of the globulin by the membrane filter (Millipore Corp.) method of Genigeorgis and Sadler (17). The same results were obtained as with the conventional fixation methods described above.

Reactions with anti-enterotoxin B in immunodiffusion. The production of enterotoxin B in *S. aureus* S6 was studied in immunodiffusion against anti-enterotoxin B serum with mechanically disrupted bacteria and digested cell walls. The enterotoxin B titer was compared with the production of extracellular enterotoxin B in the media. Approximately 1,000-fold more enterotoxin B was detected as an extracellular fraction in the media than could be detected when using digested cell walls or mechanically disrupted cells in corresponding concentrations.

**DISCUSSION**

Nonspecific reactions with *S. aureus* have often been described (2, 4-6, 9, 12, 17). We have previously reported (12) that Ig G from nonimmunized rabbits and from rabbits immunized with various bacteria reacted in immunofluorescence with *S. aureus* strains producing protein A. Pepsin digestion of the immunoglobulin preparations eliminated the reaction, showing that the Fc-fragment is involved and that the reaction is not a true antigen-antibody reaction. Since it has been shown that 98% of coagulase-positive strains of *Staphylococcus* produce protein A (11), it seemed probable that the reaction between protein A and the Fc-part of Ig G could interfere with the detection of antigens on the cell surface of *S. aureus*. Since the specific immunological activity of the immunoglobulin molecules is intact after pepsin digestion, it was suggested that the method be used to eliminate nonspecific reactions with *S. aureus* in the fluorescent-antibody technique.

Friedman and White, studying *S. aureus* strain S6, with fluorescein-labeled immunoglobulin, concluded that enterotoxin B is associated with the cell surface (16). Friedman further reported that *S. aureus*, strain S6, cells grown in the presence of Tween 80 exhibited no fluorescence when stained by the immunofluorescence technique (14). The same technique was used by Stark and Middaugh (31). They reported that *S. aureus*, strains 243 and S6, which produce enterotoxin B in food and culture media under various nutritional and environmental conditions, were rapidly demonstrated by immunofluorescence. Comparison of cell fluorescence and enterotoxin B production determined by double diffusion in gel showed that enterotoxin production could be estimated from the degree of cell fluorescence. Later, however, the degree of cell fluorescence of *S. aureus*, strains 243 and S6, was found not to correlate with enterotoxin B production in CO\(_2\) and N\(_2\) atmospheres (32). Morse and Baldwin, with conventional immunofluorescence, reported that during the transition from the exponential to the stationary phase of growth there was a rapid accumulation of both cell-associated and extracellular enterotoxin B in *S. aureus*, strain S6 (26). Extracellular enterotoxin B was synthesized until cells entered the stationary phase, during which cell-bound toxin was not detected.

Genigeorgis and Sadler (17, 18) found no correlation between gel diffusion and immunofluorescence tests for enterotoxin B. Only coagulase-negative strains were constantly negative in cell fluorescence. In attempts to produce an antiserum monospecific for enterotoxin B, they tried to dilute away "normally

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**Table 1. Immunofluorescence reaction of four strains of *S. aureus* with anti-enterotoxin B IgG and F(ab\(^1\))\(_2\)-fragments of the globulin**

| Organisms | Immunofluorescence titer/anti-enterotoxin B \(^a\) | Immunofluorescence titer/pepsin digested anti-enterotoxin B \(^d\) | Gel diffusion titer/anti-enterotoxin B \(^d\) | Protein A content/hemagglutination titer |
|-----------|-----------------------------------------------|-------------------------------------------------|------------------------------------------|------------------------------------------|
| *Staphylococcus aureus* S6 | 1/8\(^b\) | 0 | 1/160 | 1/16 |
| Cowan I | 1/160 | 0 | 0 | 1/32 |
| 4016 | 0 | 0 | 1/20 | 0 |
| Wood 46 | 0 | 0 | 0 | 0 |

\(^a\) Includes 2% anti-*Neisseria gonorrhoeae* globulin.

\(^d\) Cell fluorescence.
occurring" and minor antibodies and to adsorb sera with nonenterotoxic strains but were not able to eliminate nonspecific cell fluorescence. By disregarding cell fluorescence and noting only the brightly fluorescent precipitates that formed around enterotoxin B-producing cells, complete correlation between the results of immunofluorescence and of gel diffusion tests could be established. Since cells which did not produce toxin remained brightly fluorescent after dilution and adsorption, it was concluded that enterotoxin B is loosely associated with the cells and that when the cell does not have sufficient toxin around it, its surface antigens, being in high concentration, react with normally existing antibodies in the conjugate and stain bright green.

Our results indicate that the immunofluorescence reaction observed between anti-enterotoxin B Ig G and the cell surface of staphylococci is completely dependent on a reaction between the Fc-fragment and protein A. The results also indicate that no immunofluorescence reaction occurs with enterotoxin B on the cell surface of S. aureus under the experimental conditions used. The immunofluorescence reaction on the cell surface of S. aureus observed by us and others has been a reaction between the Fc-fragment of the Ig G molecules and protein A. The results we obtained with F(ab')2-fragments in immunofluorescence, which showed that cell-associated enterotoxin B was not present, were consistent with immunodiffusion of disrupted and digested S. aureus against anti-enterotoxin B. A 1,000-fold lower enterotoxin B content was found in digested cell walls than in the extracellular fluid, indicating that enterotoxin B is a true exotoxin present on the cell surface in low concentrations undetectable by immunofluorescence. Using FITC-labeled F(ab')2-fragments, we could also identify fluorescent precipitates of the same type as described by Genigeorgis and Sadler (17) with whole Ig G, indicating that precipitate formation is due to a specific reaction between anti-enterotoxin B and enterotoxin B.

The results of Friedman and White (16), Stark and Middaugh (32), and Morse and Baldwin (26) apparently disagree with our findings with respect to cell-associated enterotoxin B as a source of positive immunofluorescence. One of the two strains used by these authors, S6, has been investigated by us and shown to have a high content of protein A. The possibility that an immunofluorescence reaction with protein A and not with enterotoxin B has been observed must be considered. It is impossible to draw any conclusions about the effects of different environments since no study of protein A under similar conditions has been made.

This study suggests that the possibility of reactions with protein A should always be considered when working with S. aureus and immunofluorescent detection of antigens on its surface. An inhibition technique using normal Ig G not labeled with FITC would not seem to be suitable for studying specific antigen-antibody reactions on the cell surface of S. aureus since there could be steric hindrance of the blocking normal immunoglobulin molecules. It seems possible that by using F(ab')2-fragments of Ig G nonspecific reactions can be avoided and only specific antigen-antibody reactions registered.

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