Detection of Staphylococcal Exfoliative Toxin by Slide Latex Agglutination

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A simple and rapid method in which slide latex agglutination was used was developed to detect the exfoliative toxin (ET) elaborated by clinical isolates. ET types A and B (ET-A and ET-B) were purified by plate gel isoelectrofocusing, and anti-ET sera were obtained by immunizing rabbits. A specific immunoglobulin G antitoxin was then prepared from the immunized rabbit sera by fast protein liquid chromatography, and latex particles were coated with the antitoxin. Of 74 staphylococcal strains isolated from patients with staphylococcal scalded skin syndrome, 61 strains were found to produce ET by the newborn mouse bioassay. All 61 strains were shown to be positive for ET-A and ET-B production by the slide latex agglutination method. The lowest concentration of ETs detected by the latex agglutination method was 0.5 µg/ml, which was much lower than that detected by the double immunodiffusion method, with a sensitivity of 50 µg/ml. It is crucial to prove ET production by clinical isolates for the diagnosis and surveillance of staphylococcal scalded skin syndrome. The latex agglutination method is a sensitive, simple, and rapid test which can be used as an alternative to the newborn mouse bioassay.

Staphylococcus aureus is a common pathogen in human infections and causes various clinical manifestations. Disease caused by S. aureus may be the result of tissue invasion by a variety of toxins and enzymes that are elaborated by this organism. Of these extracellular products, three toxins are known that have closely related clinical features. Enterotoxins (ETs) (20, 21) cause staphylococcal scalded skin syndrome (SSSS), and toxic shock syndrome toxin (6, 15, 24) may cause toxic shock syndrome. SSSS occurs mainly in infants and children, and includes staphylococcal toxic epidermal necrolysis (TEN), Ritter's disease, staphylococcal scarlatiniform rash, and bullous impetigo (impetigo). Although SSSS is regarded as a mild disease, it is serious and sometimes fatal in newborn infants or immunocompromised patients (1, 21). To diagnose this disease accurately, it is necessary to isolate S. aureus from the patient and to demonstrate the production of ETs by the isolate.

Hitherto, ET production by S. aureus has been determined by the newborn mouse bioassay (20). This has been done by inoculating the organism into a newborn mouse and demonstrating a positive Nikolsky sign. This method is rather difficult to perform in a clinical laboratory, however, because it requires live suckling mice. Furthermore, a constant supply of such animals cannot be ensured routinely.

In this report we describe a new method for the detection of ETs from clinical isolates of S. aureus by using the slide latex agglutination method.

MATERIALS AND METHODS

Bacterial strains. Two coagulase-positive staphylococcal strains were used for the production and purification of standard ETs. Strain SA5078, which was obtained from a patient with impetigo and classified into phage group 2 (lysed by phages 55 and 71), was used for ET type A (ET-A) purification. Strain SA5079, which was isolated from a patient with TEN and classified into phage groups 1 and 3 (lysed by phages 29, 47, 54, 75, 79, and 80), was used for ET type B (ET-B) purification. It was shown by I. Kondo (Department of Bacteriology, Tokyo Jikei University School of Medicine, Tokyo, Japan) that strains SA5078 and SA5079 produce ET-A and ET-B respectively.

A total of 74 strains of S. aureus were isolated from patients who were diagnosed with SSSS (26 patients with TEN and 48 patients with impetigo) in the Asahikawa Medical College Hospital and affiliated hospitals during an 8-year period from 1977 to 1984. There were no duplicate strains from the same patients. The isolates were kept freeze-dried until they were tested.

Purification of ETs. Strains SA5078 and SA5079 were cultured in TY medium (10 g of yeast extract, 17 g of Trypticase [BBL Microbiology Systems, Cockeysville, Md.], 5 g of NaCl, and 2.5 g of K2HPO4 in 1 liter of distilled water [18]) under conditions of continuous agitation with a magnetic stirrer at 37°C for 48 h in 10% CO2. Cultures were centrifuged at 6,800 × g for 40 min, and the supernatants were concentrated by ammonium sulfate precipitation with various concentrations of (NH4)2SO4, as described by Kondo et al. (18, 19). The resultant precipitates were dialyzed against distilled water. Samples were then subjected to preparative thin-layer isoelectrofocusing in a granulated gel with a pH gradient of 3.5 to 9.5 (26). After isoelectrofocusing, protein bands were located by the zymogram print method (26), and discrete bands of toxin were scraped from the plate. Purified ETs were eluted from the gel with distilled water.

The molecular weights of the purified ETs were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12), and the quantity of protein was determined by the assay described by Bradford (7), with bovine serum albumin used as a standard.

Production of antiserum to ET. Antisera were produced in rabbits by repeatedly injecting purified ETs. A total of 0.5 mg of ET in 0.5 ml of phosphate-buffered saline mixed with 0.5 ml of Freund complete adjuvant was given subcutaneously at 2-week intervals. Five doses were given, and 3 weeks after the last injection, the animals were exsanguinated.
TABLE 1. Specific activities of ET-A and ET-B at various stages of purification

<table>
<thead>
<tr>
<th>Purification stage and ET</th>
<th>ET activity (EU/ml)</th>
<th>Protein (mg/ml)</th>
<th>Sp act (EU/mg)</th>
<th>Purification index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-A</td>
<td>40</td>
<td>0.08</td>
<td>500</td>
<td>1</td>
</tr>
<tr>
<td>ET-B</td>
<td>20</td>
<td>0.04</td>
<td>500</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-A</td>
<td>2,560</td>
<td>2.8</td>
<td>914</td>
<td>1.8</td>
</tr>
<tr>
<td>ET-B</td>
<td>2,560</td>
<td>1.8</td>
<td>1,422</td>
<td>2.8</td>
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<tr>
<td>Isoelectro focusing</td>
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</tr>
<tr>
<td>ET-A</td>
<td>1,280</td>
<td>0.2</td>
<td>6,400</td>
<td>12.8</td>
</tr>
<tr>
<td>ET-B</td>
<td>40</td>
<td>0.005</td>
<td>8,000</td>
<td>16.0</td>
</tr>
</tbody>
</table>

Isolation of anti-ET immunoglobulin G. Anti-ET sera were concentrated by ammonium sulfate precipitation at 33% saturation. The precipitate was dialyzed against a 0.9% NaCl solution for 48 h and then underwent fast protein liquid chromatography (Mono Q column; Pharmacia Fine Chemicals, Piscataway, N.J.; initial buffer, 0.067 M Tris phosphate buffer [pH 8.6]; final buffer, 0.5 M Tris phosphate buffer [pH 4.2]), and an immunoglobulin G (IgG) fraction was eluted. The fraction was confirmed as IgG by the immunodiffusion method (23) by using standard anti-rabbit sera.

Preparation of the latex reagent. Polystyrene latex particles (diameter, 0.81 μm; Difco Laboratories, Detroit, Mich.) were diluted in glycine-buffered saline (GBS; 7.3 g of glycine, 10 g of NaCl in 1 liter of distilled water [pH 8.2]) and were mixed with anti-ET IgG diluted in GBS at various concentrations, to determine the optimal concentration for the slide latex agglutination test. The mixed suspension was incubated at 37°C for 2 h and centrifuged at 700 × g for 20 min. The latex particles that were coated with the specific IgG were washed twice with GBS and suspended in GBS containing 0.5% bovine serum albumin, resulting in a final latex concentration of 1% (wt/vol). The reagent was stored at 4°C.

Biological assay for ET. At each stage of purification, the ETs were serially diluted and subcutaneously injected into 1- to 3-day-old newborn mice. The activity of each sample was expressed in terms of exfoliating units (EUs). The titer of each sample was the reciprocal of the highest dilution that gave a positive Nikolsky sign (extensive exfoliation by light stroking with a finger) in newborn mice (10, 18, 22).

Detection of ET from clinical isolates. For the newborn mouse bioassay, which was done by the method described by Melish and Glasgow (20), 0.1 ml of a 24-h culture of the test organism grown in TY medium (10^8 CFU) was inoculated subcutaneously into newborn mice. The organism, which caused a positive Nikolsky sign within 16 h, was regarded as an ET-producer strain. For the double immunodiffusion test (23), 10-fold concentrates of the culture supernatant of the organism were tested against anti-ET-A and anti-ET-B sera on an agar plate. If a precipitation line was formed between the wells, which were filled with the concentrates and antisera, the organism was regarded as an ET producer. For the slide latex agglutination test, 50 μl of the culture supernatant of the test organism and 50 μl of the latex reagent for ET-A or ET-B were mixed on a glass slide by tilting. If agglutination was visible within 3 min, the organism was regarded as an ET producer.

RESULTS

Purification of ETs and their properties. The activities of the ET-A and ET-B preparations at various stages of purification are shown in Table 1. In the culture supernatant, the specific activity was 500 EU/mg of protein for both ET-A and ET-B. After isoelectrofocusing, the specific activity was 6,400 EU/mg of protein for ET-A and 8,000 EU/mg of protein for ET-B. The results of preparative thin-layer isoelectrofocusing of ET-A and ET-B are shown in Fig. 1. The isoelectric points of both ET-A and ET-B were 7.0. When subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, ET-A and ET-B each migrated as a single band of homogeneous protein, with molecular weights of about 30,900 for ET-A and about 30,200 for ET-B (data not shown).

Preparation of the latex reagent and its sensitivity. A fast protein liquid chromatographic system was used to isolate IgG from anti-ET rabbit sera. Two major peaks were obtained, and both fractions were found to be IgG. Only the second peak fraction had anti-ET activity, however, which was collected and used.

The optimal concentration of anti-ET IgG in the latex suspension was sought. A ratio of 10 to 20 μg of IgG to 1 mg of latex was found to be the most suitable. With lower

FIG. 1. Isoelectrofocusing of ET-A (A) and ET-B (B) by preparative thin-layer isoelectrofocusing. OD280, Optical density at 280 nm.
concentrations, the agglutination was barely visible, and with higher concentrations, spontaneous agglutination was observed. In this study, we used 200 μg of specific IgG per ml of 1% latex suspension to coat the latex particle (Fig. 2).

The lowest concentration of ET identified by this latex reagent was 0.5 μg/ml, while that identified by the double immunodiffusion method with anti-ET sera was 50 μg/ml.

**Detection of ET production in clinical isolates.** Seventy-four clinical isolates from patients with SSSS were compared for ET production by the newborn mouse bioassay, the double immunodiffusion method, and the latex agglutination method. Of the 74 strains, 61 were found to produce ETs by the newborn mouse bioassay. These 61 strains were positive for ET-A, ET-B, or both by the latex agglutination method. Conversely, the remaining 13 strains that did not produce ETs by the newborn mouse bioassay were negative for both ET-A and ET-B by the latex agglutination method. By using the double immunodiffusion method, 59 of 74 strains were found to produce ET-A, ET-B, or both. Of the 26 strains that caused TEN, 24 strains were detected by each of the detection methods: the newborn mouse bioassay, the double immunodiffusion method, and the latex agglutination method. Of the 48 strains that caused impetigo, 37, 35, and 37 strains were detected by the three methods, respectively. The types of ET determined by the double immunodiffusion and latex agglutination methods are given in Table 2. Using the double immunodiffusion method, 19 of 59 strains produced ET-A, 31 produced ET-B, and 9 produced both ET-A and ET-B. With the latex agglutination test, 15 of 61 strains produced ET-A, 32 produced ET-B, and 14 produced both ET-A and ET-B.

Of the 15 ET-negative strains determined by double immunodiffusion, 2 were identified as serotype B by the latex agglutination method. A total of 4 of the 19 serotype A strains and 1 of the 31 serotype B strain determined by the immunodiffusion method were classified as serotype A and B by the latex agglutination method.

**DISCUSSION**

In 1970, Melish and Glasgow (20) proposed SSSS as a clinical entity which includes Ritter’s disease, TEN, staphylococcal scarlatiniform rash, and impetigo. They demonstrated that all staphylococci isolated from patients with these diseases belong to phage group 2, and when they were inoculated into newborn mice, all showed a positive Nikolsky sign. Afterward, it was found that the staphylococci that were isolated from such patients elaborate an exotoxin, ET, that is regarded as the pathognomonic agent for SSSS (22). Research was carried out to purify the ET (3, 10, 16, 18, 22), and it was demonstrated that there are two distinct serotypes of ET, ET-A and ET-B (2, 17, 19). In a recent report by Bailey et al. (4) and a review by Freer and Arbuthnot (14), the isoelectric point of ET-A was 7.0 and that of ET-B was 6.95; and their molecular weights were 30,000 and 29,500, respectively. Each value was almost identical to those that we obtained by preparative thin-layer isoelectrofocusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

We used a slide latex agglutination method with the culture supernatant of S. aureus to identify the ET production of the organism. Latex particles were coated with specific IgG from immunized rabbit serum made from purified ET-A and ET-B. This method was almost 100 times as sensitive as the double immunodiffusion method. All strains that were positive for ET by the newborn mouse bioassay (20) were also positive for ET-A, ET-B, or both by the slide latex agglutination test.

Of 74 isolates from patients who were diagnosed with clinical SSSS, 61 (82%) were found to produce ET. This rate of ET-positive strains might not be high enough, in view of the definition of SSSS, namely, that this clinical entity is caused by ET-producing staphylococci. It is likely that some of the isolates in this series were resident rather than causative organisms. Therefore, to identify the causative staphylococci of SSSS, screening of the isolates for ET production is essential. During an epidemiological investigation of an outbreak of SSSS (8, 11), a microbiological study must include tests for ET productivity other than phage typing, since ET-positive strains are not always phage group 2 staphylococci (9, 13, 25).

The slide latex agglutination method for the detection of ET-A and ET-B is a sensitive, simple, and rapid test. It could be used to test a large number of isolates as an alternative to the newborn mouse bioassay or the double immunodiffusion method.

**ACKNOWLEDGMENTS**

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