QUALITATIVE AND QUANTITATIVE METHODS FOR DETECTING STAPHYLOCOCCAL EPIDERMOLYTIC TOXIN

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PLATES XIII-XV

It is now generally accepted that strains of \textit{Staphylococcus aureus} isolated from patients with staphylococcal toxic epidermal necrolysis of Ritter's type (TEN) or staphylococcal bullous impetigo produce an exotoxin, termed epidermolytic toxin, that is responsible for the intra-epidermal splitting in the plane of the stratum granulosum that characterises these lesions. In TEN, large areas of skin are affected and the loosened epidermis peels extensively. The discovery of epidermolytic toxin followed the important observation of Melish and Glasgow (1970) that staphylococci isolated from patients with TEN or bullous impetigo caused extensive epidermal splitting when injected into newborn mice. Subsequent work has been concerned mainly with the isolation and partial characterisation of the epidermolytic toxin (Arbuthnott \textit{et al.}, 1971; Kapal and Miller, 1971; Arbuthnott \textit{et al.}, 1972; Melish, Glasgow and Turner, 1972; Kondo, Sakurai and Sarai, 1973 and Arbuthnott, Billcliffe and Thompson, 1974; Kondo \textit{et al.}, 1975) and with studies of the mode of action of epidermolytic toxin (Kapral and Miller, 1972; Lillibrige, Melish and Glasgow, 1972; McCallum, 1972; Arbuthnott, Kent and Noble, 1973; Elias \textit{et al.}, 1974a and b; Wiley \textit{et al.}, 1974; McLay, Arbuthnott and Lyell, 1975).

The toxin is a relatively thermostable antigenic protein with a molecular weight of approximately 25,000. The preparative isoelectric focusing studies of Arbuthnott \textit{et al.} (1974) showed that the main form of epidermolytic toxin has $pI = 7.0$; a minor component having a $pI = 6.0$ was detected. In thin-layer-gel isoelectric focusing the main form exhibited complex microheterogeneity, and up to seven protein bands were detected between $pH 6.2$ and $7.0$. Recently, Japanese workers have described the isolation of two serologically distinct forms termed A and B toxins (Kondo \textit{et al.}, 1974 and 1975), and the studies of Arbuthnott \textit{et al.} (1974) also suggested the existence of two antigenically distinct forms of the toxin.

The present study was undertaken with the aim of assessing the specificity and sensitivity of in-vitro methods for detecting and measuring epidermolytic toxin production by \textit{S. aureus}. The strains were provided by Dr Elizabeth H.

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Asheshov, Central Public Health Laboratory, Colindale, London NW9 5HT, and included some likely producers of epidermolytic toxin and also a number of strains selected at random from an on-going survey of staphylococci isolated from hospital infections. In a preliminary test, culture supernates were concentrated and screened for epidermolytic toxin by biological testing in 3-day-old mice, by double diffusion tests against antibody to purified epidermolytic toxin raised in rabbits, and by the appearance of a characteristic pattern of protein bands in thin-layer-gel isoelectric-focusing tests. Thereafter, the amount of epidermolytic toxin present in supernates was estimated quantitatively by titration in 3-day-old mice and by a radial-immunodiffusion test.

**Materials and methods**

**Strains of S. aureus.** Ninety-eight strains of *S. aureus* were supplied by Dr Asheshov. They had been phage typed in the Cross-Infection Reference Laboratory, Central Public Health Laboratory, Colindale, with the International Basic Set of typing phages at routine test dilution (RTD) and at RTD x 100. They were kept on slopes of Columbia Agar Base (Oxoid) at 4°C and were subcultured at monthly intervals. Screening tests for production of epidermolytic toxin were performed without prior knowledge of the phage-typing patterns.

**Production of epidermolytic toxin.** Each strain was plated on horse-blood agar (Columbia Agar Base containing 10% (v/v) of horse blood (Tissue Culture Services, Slough, Buckinghamshire)) and 75-ml volumes of a medium containing Casamino acids and yeast diffusate (Bernheimer and Schwartz, 1963) in 250-ml flanged Erlenmeyer flasks were inoculated from single colonies. Cultures were incubated overnight at 37°C in an IH 400 orbital shaking incubator (Gallenkamp, Technico House, London). After centrifugation (10 000 g for 30 min. at 4°C), a sample of supernate was kept for reference and the remainder dialysed against 90% saturated ammonium sulphate at 4°C. The sediment, harvested by centrifugation at 48 000 g for 30 min., was dissolved in one-tenth of the original volume in 0·015M phosphate-buffered saline, pH 7·2, and dialysed against this buffer to remove residual ammonium sulphate. Supernates and concentrates were stored at -20°C.

**Purification of epidermolytic toxin.** Toxin was purified by preparative isoelectric focusing (Arbuthnott et al., 1974).

**Haemolytic activity** was measured by titration against 0·8 (v/v) rabbit RBC (McNiven, Owen and Arbuthnott, 1972).

**Protein estimations.** Protein was estimated by the method of Lowry et al. (1951) with bovine serum-albumin fraction V (Armour Pharmaceutical Co. Ltd, Eastbourne, Sussex) as standard.

**Screening tests for epidermolytic toxin production**

**Mouse test.** After neutralising the α-toxin content of concentrates by adding a slight excess of standard α-antitoxin (CPP 76/73, Wellcome Research Laboratories, Beckenham, Kent), groups of five 3-day-old CFLP mice (weight 2·0 g) were challenged, each animal receiving 0·05 ml subcutaneously. The presence of epidermolytic toxin was indicated by the appearance after 3-6 h of the Nikolsky sign (loosening of the epidermis detected after stroking or gently pinching the skin).

Each strain was screened also for the ability to produce epidermolytic toxin *in vivo.* Cells from overnight static cultures in Oxoid Nutrient Broth were washed three times (3000 g for 20 min. at room temperature) with Peptone Water (Oxoid), and suspensions containing c. 1 x 10^9 c.f.u. per ml by viable count (Miles, Misra and Irwin, 1938) prepared in Peptone Water. Groups of four 3-day-old mice were challenged subcutaneously, each animal receiving 0·05 ml of cell suspension (5 x 10^8 c.f.u.). The animals were tested for the appearance of a positive Nikolsky sign 3 and 6 h after challenge.

**Double-diffusion tests.** Concentrates were tested against an antiserum (no. AS123A)
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raised in rabbits against purified epidermolytic toxin prepared from strain 123A (Arbuthnott et al., 1974). Wells 2-5 mm in diameter and 9 mm centre to centre were cut in plates (8·5 cm x 9·5 cm) coated with 10 ml of 1 % (w/v) agarose (L'Industrie Francaise) in saline.

Thin-layer-gel isoelectric focusing. Isoelectric focusing in thin layers of polyacrylamide gel (Vesterberg, 1972; Davies, 1975) was carried out by means of the Multiphor apparatus (LKB Instruments Ltd, Croydon, Surrey). Samples of concentrate (10 μl) were applied to polyacrylamide gel (Hjerten values: T = 5; C = 3) containing 2·4 % (w/v) of Ampholine, pH 3·5–10·0. A potential of 24 V per cm was applied at 30 mA and was increased in steps to 100 V per cm after 70 min.; the temperature was maintained at 8°C. Isoelectric focusing was continued for a total period of 2 h. The pH across the gel was then measured at intervals of 0·5 cm with an antimony surface electrode assembly (Activion, Kinglassie, Fife) coupled to a PHM26 pH meter (Radiometer, Copenhagen, Denmark). Protein bands were detected by staining with an acidic extract of Coomassie Brilliant Blue R250 (BDH, Poole, Dorset) at room temperature (LKB Application Note no. 149, 1974).

Quantitative tests performed on supernates

Radial-immunodiffusion test. Plates (8·5 cm x 9·5 cm) were coated with 10 ml of Agarose gel (Agarose 1·5 % (w/v) in 0·1 M sodium barbitone buffer, pH 7·2, with sodium azide 0·1 % (w/v) added as preservative) containing antiserum (AS123A) at a final dilution of 1 in 30. Wells (2·0 mm in diameter) contained 4-μl volumes of serial doubling dilutions of culture supernate. Plates were incubated overnight at room temperature in a moist chamber, and the diameters of rings of immunoprecipitate were measured by means of a calibrated rule (Hoechst, Beringwerke). The concentration of epidermolytic toxin present was determined from a standard curve of ring diameter squared (D2) plotted against protein concentration for highly purified epidermolytic toxin.

Bioassay in newborn mice. Each of a series of graded dilutions of supernate were tested by injection of 0·05 ml subcutaneously in groups of four 3-day-old mice. A mouse unit was defined as the highest dilution that gave a positive Nikolsky sign in two or more animals within 6 h.

RESULTS

Production of epidermolytic toxin by strains of S. aureus

The presence of epidermolytic toxin in concentrates prepared from culture supernates of 98 strains of S. aureus by ammonium-sulphate precipitation was assessed by three criteria: the ability to cause epidermal splitting in newborn mice, the formation of a line of precipitate against an antiserum prepared against highly purified epidermolytic toxin, and the appearance of a characteristic pattern of protein bands in thin-layer-gel isoelectric focusing in the region pH 6·0–7·0 (fig. 1). Repeated testing showed that 11 of the strains gave positive results in all three tests. Two strains, when first tested, gave positive results only in the newborn mice; after subculture these strains were found to be negative in all screening tests and it seems likely that these were very weak toxin producers.

After completion of the screening tests, the phage-typing patterns were supplied by Dr Asheshov. Of the 11 strains that gave positive results in all three tests, eight (73 %) belonged to phage-group II; seven of these were lysed by phage 71 (at RTD) and one by phage 3A (at RTD x 100). The three producer strains that did not belong to group II comprised one group-III strain, one strain having the typing pattern 80/54/85+ and an untypable strain. The typing patterns of the 11 positive strains were confirmed by re-testing and are
TABLE I

Properties of eleven epidermolytic strains of Staphylococcus aureus

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Phage-typing pattern</th>
<th>Haemolysin titre (units per ml)</th>
<th>Amount of epidermolytic toxin estimated by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>bioassay in newborn mice (mouse units per ml)</td>
</tr>
<tr>
<td>1</td>
<td>71</td>
<td>256</td>
<td>3800</td>
</tr>
<tr>
<td>8</td>
<td>71</td>
<td>32</td>
<td>1800</td>
</tr>
<tr>
<td>29</td>
<td>71</td>
<td>32</td>
<td>2100</td>
</tr>
<tr>
<td>46</td>
<td>3A</td>
<td>16</td>
<td>400</td>
</tr>
<tr>
<td>47</td>
<td>54/85</td>
<td>128</td>
<td>1320</td>
</tr>
<tr>
<td>48</td>
<td>71</td>
<td>128</td>
<td>3480</td>
</tr>
<tr>
<td>53</td>
<td>71</td>
<td>128</td>
<td>3680</td>
</tr>
<tr>
<td>55</td>
<td>80/54/85/+</td>
<td>128</td>
<td>2520</td>
</tr>
<tr>
<td>86</td>
<td>71</td>
<td>256</td>
<td>5040</td>
</tr>
<tr>
<td>96</td>
<td>71</td>
<td>128</td>
<td>520</td>
</tr>
<tr>
<td>100</td>
<td>NT</td>
<td>&lt;2</td>
<td>200</td>
</tr>
</tbody>
</table>

included in table I. With reference to the frequency of toxin production by group-II strains, it is notable that the strains examined included a total of 14 strains lysed by group-II phages of which eight (57%) proved to be producers of epidermolytic toxin and that all the "type 71" strains were toxigenic. All 98 strains were screened also for their ability to produce epidermolytic toxin in vivo by challenging newborn mice subcutaneously with a large dose of viable organisms (5x10⁸ c.f.u.) The 11 strains that produced epidermal splitting under these conditions were the 11 strains found to be positive by the criteria mentioned above; the two atypical strains gave negative results in this test. The in-vitro screening tests, therefore, provided an accurate indication of the toxigenicity of the strains in the present survey.

Quantitative tests for epidermolytic toxin

To measure the amount of epidermolytic toxin produced by the 11 positive strains, culture supernates were titrated for epidermolytic toxin activity in newborn mice. Activities ranged from 200 to 5000 mouse units per ml. Although the newborn-mouse test is highly specific, it requires large numbers of 3-4-day-old animals. When large numbers of preparations of unknown potency have to be tested, as in purification studies, the test is inconvenient and expensive. For this reason it was decided to assess the value of a radial-immunodiffusion assay against antitoxin AS123A. The specificity of this serum was found to be adequate for this purpose; in double-diffusion tests with concentrates from all 98 strains, distinct lines of precipitate were obtained only with strains that had proved positive by other criteria, and rings of immunoprecipitate were obtained in radial-immunodiffusion tests only with positive strains. Standard solutions of highly purified epidermolytic toxin containing 0-25–1-0 mg of toxin per ml were included in each experiment and were used
to compile a standard curve of $D^2$ plotted against toxin concentration from which the potencies of test samples were determined. A correlation curve (fig. 2) of epidermolytic-toxin levels determined by radial immunodiffusion and by titration in newborn mice for supernates from the 11 positive strains shows that, with the exception of strain 53, good correlation was obtained. The radial-immunodiffusion assay is recommended for rapid quantitative screening of samples containing more than 200 μg of epidermolytic toxin per ml. When information obtained from the radial-immunodiffusion test is used, the number of newborn mice required to determine the potency in mouse units can be reduced considerably.

**Evidence for serologically distinct forms of epidermolytic toxin**

In radial-immunodiffusion tests it was observed that supernates prepared from six of the 11 positive strains gave two rings while the remainder gave single rings of immunoprecipitate (fig. 3). The diameter of the inner ring relative to the outer ring varied, and in one case (strain 53) the diameters of the two rings were almost equal. (Only the diameter of the outer ring was used in calculating the toxin concentration by radial immunodiffusion assay.) These observations suggested that antiserum AS123A contained antibodies to two serologically distinct forms of epidermolytic toxin and that strains differed in the relative amounts of these components that they produced. Double-diffusion tests were performed to investigate the serological relationship between the toxin preparations from the 11 positive strains and the reference strain 123A (fig. 4).

![Graph showing correlation between bioassay and radial-immunodiffusion test](image)

**Fig. 2.—** Correlation between the bioassay of toxin in newborn mice (mouse units per ml) and the radial-immunodiffusion test (mg per ml) for culture supernates prepared from 11 toxinogenic strains. Strain numbers are shown adjacent to open circles.
Table II

Comparison of protein bands at pH 7.0 in thin-layer-gel isoelectric focusing and number of antigens detected by double diffusion tests with unabsorbed and absorbed antiserum (AS123A)

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Protein bands at c. pH 7.0 in gel isoelectric focusing</th>
<th>Number of antigens detected by double diffusion tests with antisera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AS123A</td>
</tr>
<tr>
<td>1</td>
<td>ii and trace of i</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>i and ii</td>
<td>2</td>
</tr>
<tr>
<td>29</td>
<td>i and ii</td>
<td>2</td>
</tr>
<tr>
<td>46</td>
<td>i</td>
<td>1</td>
</tr>
<tr>
<td>47</td>
<td>i</td>
<td>1</td>
</tr>
<tr>
<td>48</td>
<td>i</td>
<td>1</td>
</tr>
<tr>
<td>53</td>
<td>i and ii</td>
<td>2</td>
</tr>
<tr>
<td>55</td>
<td>i</td>
<td>1</td>
</tr>
<tr>
<td>86</td>
<td>i and ii</td>
<td>2</td>
</tr>
<tr>
<td>96</td>
<td>i and ii</td>
<td>2</td>
</tr>
<tr>
<td>100</td>
<td>i</td>
<td>1</td>
</tr>
<tr>
<td>123A</td>
<td>i and ii</td>
<td>2</td>
</tr>
</tbody>
</table>

* Antiserum AS123A(47) was prepared by absorbing serum AS123A (0.6 ml) with concentrate from strain 47 (0.15 ml).
† Antiserum AS123A(1) was prepared by absorbing serum AS123A (0.6 ml) with concentrate from strain 1 (0.06 ml). These mixtures were incubated at 37°C for 90 min. and centrifuged (20000 g for 30 min.) before testing.
†† nt = Not tested.

The results of these tests, considered along with the ring patterns obtained in radial-immunodiffusion tests, indicate that the line of precipitate obtained with concentrates from strains 29, 53, 86, and 96 was in fact, a "double" line and that these strains, together with strains 1 and 8 which gave two distinct lines of precipitate, produced two antigenic components. In contrast, strains 46, 47, 84, 55 and 100 produced only one of these antigens. Moreover, the double-diffusion pattern obtained with strain 1 (fig. 4b) indicated that the two components were serologically distinct. Also in fig. 1 it can be seen that in thin-layer-gel isoelectric focusing the concentrates from strains that produced both antigenic forms of epidermolytic toxin showed the presence of two protein bands (bands i and ii) in the region pH 7.0-7.2), whereas those from strains that produced only one form showed only the more cationic of these bands (band i). None of the concentrates contained band ii in the absence of band i, but in the concentrate from strain 1 band ii was present in considerable excess over band i. These findings are summarised in table II. Antiserum AS123A absorbed with concentrate from strain 47 and tested by double diffusion against concentrates from 10 of the 11 positive strains and reference strain 123A gave no visible line of precipitate with strains 46, 47, 48, 55 and 100. Conversely, serum AS123A absorbed with concentrate from strain 1 gave a single line of precipitate against concentrates prepared from all 11 strains (table II). Thus all the toxigenic strains produced epidermolytic toxin designated serotype i and six of these (54%) produced also epidermolytic toxin designated serotype ii.
Preparative isoelectric focusing of epidermolytic toxin from strain 47

Previous preparative isoelectric-focusing studies of toxin prepared from strain 123A (Arbuthnott et al., 1974) revealed the presence of a major component designated ETA having a pI = 7·0 and a minor component ETB having a pI = 6·0. To determine whether both ETA and ETB were present in strains known to produce only epidermolytic toxin of serotype i, preparative isoelectric focusing was performed with toxin prepared from strain 47. From fig. 5 it can be seen that the main peak of epidermolytic toxin activity as assessed by radial immunodiffusing had a pI = 7·0 and that a second smaller peak was also detected having a pI = 6·0. These components correspond closely to ETA and ETB produced by strain 123A.

Heat resistance of epidermolytic toxin

High-potency purified epidermolytic toxin (ETA) from strain 123A containing 16 000 mouse units per ml, when heated for 30 min. at 60°C, lost 70% of its biological activity. However, heating at 100°C for 30 min. did not cause a further reduction in activity. A substantial proportion of activity, therefore,
was thermostable. Heating to 60°C for 30 min. also led to disappearance of band ii from the protein-band pattern seen in thin-layer-gel isoelectric focusing; this was accompanied by the appearance of a single ring rather than a double ring of immunoprecipitate in radial-immunodiffusion tests. When heated at 100°C for 10 or 30 min. the band pattern in thin-layer isoelectric-focusing gels changed markedly with the appearance of progressively more acidic components. Preliminary results obtained with culture supernates prepared from the 11 positive strains isolated in this survey showed that in all cases the titre of epidermal splitting activity was not reduced by heating for 30 min. at 60°C. This suggests that the heat resistance of epidermolytic toxin is more marked in culture supernates than in purified preparations.

DISCUSSION

In the first study of the epidermolytic activity of S. aureus, Melish and Glasgow (1970) found that all of 17 phage-group-II strains isolated from patients with staphylococcal scalded-skin syndrome and two group-II strains isolated respectively from cases of otitis media and oesophageal ulcer produced epidermal splitting. In contrast, none of 20 non-group-II strains exhibited epidermolytic activity. Melish and Glasgow (1970) failed to detect epidermolytic toxin production in culture in vitro.

Working with isolates from exfoliative lesions, which included strains from the study of Melish and Glasgow (1970), Kapral and Miller (1971) demonstrated epidermolytic activity in 13 of 15 strains when viable organisms were injected into animals, but only 6 (40%) of the strains produced detectable amounts of epidermolytic toxin in vitro. In a later survey of 200 randomly selected phage-group-II strains, Kapral (1974) detected epidermolytic toxin in filtrates of 40% of cultures; he also reported epidermolytic toxin production in 2% of 1000 strains of non-group-II staphylococci. Recently, Kondo et al. (1974) isolated two serologically distinct forms—"exfoliatin A" and "exfoliatin B"—of epidermolytic toxin. The Japanese workers have since screened for the distribution of the A and B forms among 43 strains of S. aureus isolated from patients with impetigo or Ritter's disease. Nine strains (20%) produced only "exfoliatin A", 15 strains (35%) produced only "exfoliatin B", 16 strains (37%) produced both and three strains (7%) failed to produce toxin in vitro. It is of particular interest that there was no correlation between phage-typing pattern and the serotype of toxin produced, and that 19 strains (44%) belonged to phage groups other than II.

When making surveys of the distribution among strains of the ability to produce epidermolytic toxin in culture, and of the amount of toxin formed and its serotype, it is desirable that toxin production in vitro should reflect as closely as possible toxigenicity in vivo. In the present study, the results of three screening tests for toxin in concentrated supernates were well correlated with the results obtained by injecting viable organisms into newborn mice. Of the three screening tests employed to analyse concentrates, the newborn-
mouse test was the most sensitive; 13 concentrates were positive in this test, whereas the double-diffusion test and analysis of protein-band patterns by thin-layer-gel isoelectric focusing gave positive results for 11 of these. It should be pointed out, however, that concentrates prepared after subculturing the anomalous strains were consistently negative. In future surveys, therefore, the existence of strains with weak or variable toxin production may have to be taken into account.

SEROLOGICAL ANALYSIS OF CULTURE FLUIDS BY DOUBLE-DIFFUSION AND RADIAL-IMMUNODIFFUSION TESTS should prove particularly valuable in both qualitative and quantitative assessment of toxin production. However, the appearance of a single line of precipitate in double-diffusion tests does not necessarily indicate the presence of a single antigenic type of epidermolytic toxin, because four of the 11 positive strains that gave a single line were shown by radial immunodiffusion and gel-isoelectric focusing to produce two serotypes.

The reasonably close correlation between the results obtained by the radial-immunodiffusion test and the newborn-mouse test in measuring the potency of toxin preparations from the 11 positive strains indicates that the former should prove extremely useful for assaying culture fluids and fractions obtained in purification studies. Also, the use of sera specific for serotype-i or serotype-ii toxins will make it possible to estimate individual serotypes in fluids that contain both.

Although not designed primarily as a study of the frequency of toxin production by staphylococci of particular phage groups, this investigation lends support to the view that epidermolytic toxin production is not a uniform property of all phage-group-II strains (Kapral, 1974) and that the production of the toxin is not restricted to phage-group II (Kapral, 1974; Kondo et al., 1975). These findings, and particularly the observation of the Japanese workers that 41% of epidermolytic strains of S. aureus did not belong to phage-group II, suggest that surveys of epidermolytic toxin production by staphylococcal strains isolated in other countries might yield interesting results.

RESULTS OBTAINED BY PREPARATIVE ISEELECTRIC FOCUSING OF EPIDERMOLYTIC TOXIN prepared from a strain that produced only toxin of serotype i support the previous suggestion (Arbuthnott et al., 1974) that epidermolytic toxin exhibits two types of molecular heterogeneity. However, because toxin prepared from this organism contained components $\text{ET}_A$ ($\rho I = 7.0$) and $\text{ET}_B$ ($\rho I = 6.0$), the findings do not uphold our earlier suggestion that $\text{ET}_A$ and $\text{ET}_B$ are serologically distinct. This discrepancy can be explained by the failure to resolve the serologically distinct components by preparative isoelectric focusing in a broad $pH$ gradient. This is not surprising, because gel-isoelectric focusing suggests that bands i and ii differ only slightly in charge. Considerable further study is required to elucidate the inter-relationship between different molecular forms of epidermolytic toxin.

In preliminary experiments, analysis of preparations of heated purified epidermolytic toxin by serological and analytical isoelectric-focusing methods suggests that a detailed investigation of the effect of heat on the toxin may provide an experimental approach to this problem.
SUMMARY

Concentrated supernates of cultures of 98 strains of *Staphylococcus aureus* were screened for the production of epidermolytic toxin by (1) biological tests in 3-day-old mice, (2) double-diffusion precipitation tests against specific antiserum, and (3) the appearance of characteristic protein bands on thin-layer-gel isoelectric focusing. Positive results were obtained in all three of these tests with supernates from 11 of these cultures; the same 11 strains, and no others, produced epidermal splitting when newborn mice were challenged with viable organisms. Of the 14 phage-group-II strains included in the survey, eight (58%) produced epidermolytic toxin. Three toxinogenic strains belonged to phage groups other than group II.

A radial-immunodiffusion test employing antiserum to purified epidermolytic toxin proved satisfactory for measuring amounts of epidermolytic toxin in excess of 200 µg per ml. The results of immunodiffusion tests indicated that six of the 11 positive strains produced two serologically distinct forms of epidermolytic toxin and that the remainder produced only one of these. A striking correlation was observed between the presence of toxin of serotypes i and ii and the occurrence of protein bands i and ii in thin-layer isoelectric-focusing gels.

These tests should facilitate the qualitative and quantitative assessment of the production of different serotypes of epidermolytic toxin by *S. aureus* in future surveys of strains isolated from toxic epidermal necrolysis of Ritter's type and impetigo.

The two forms of epidermolytic toxin previously designated ET_A (pI = 7.0) and ET_B (pI = 6.0) were detected by preparative isoelectric focusing of serotype-i toxin. Evidence suggests that studies of the effect of heat should provide a means of investigating the relationship between the different molecular forms of epidermolytic toxin.

We thank Dr Elizabeth H. Asheshov for selecting the staphylococcal isolates and for performing the phage-typing, and Dr A. Lyell for helpful criticism.

REFERENCES


Fig. 1.—Thin-layer gel isoelectric focusing (see Materials and Methods) of concentrates prepared from epidermolytic strains of Staphylococcus aureus showing the distribution of protein bands i, ii, and iii. Samples A (purified toxin from strain 123A) and B (enzyme from strain 123B) were included for reference. Strain numbers are shown below each track.
Fig. 3.—Radial-immunodiffusion tests in Agarose containing antiserum AS123A showing single and double ring patterns obtained with culture supernates from epidermolytic strains of *S. aureus*. Strain numbers are shown below each well. Strains 9 and 30 were negative in this test; Bl = blank well.
Fig. 4—Double diffusion tests in which concentrates prepared from epidermolytic strains of S. aureus were tested against antiserum AS123A: (a) pattern obtained with concentrates from strains 123A, 48, 1, 46, 8 and 100; (b) pattern obtained with concentrates from strains 123A, 48, 96, 47 and 53.
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