SHORT ARTICLES

CELL-ASSOCIATED ALPHA-TOXIN FROM STAPHYLOCOCCUS AUREUS

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The relationship between intracellular and extracellular bacterial toxins was recently reviewed by Raynaud and Alouf (1970). This aspect has been extensively investigated in the case of the clostridial toxins, diphtheria toxin, cell-associated toxins of Gram-negative bacteria and the enterotoxin B of Staphylococcus aureus.

In comparison, relatively little is known of the haemolytic toxins of Staph. aureus. This may be due to the fact that Raynaud et al. (1955), using ultrasonic disintegration, ionic shock, penicillin-induced lysis and mechanical agitation for cell disruption, failed to detect cell-associated activity. More recently Hendricks and Altenbern (1968) detected very small quantities of haemolysin in lysates produced by lysostaphin treatment. They reported that the haemolysin was synthesised and released continuously into the culture medium and that small alkaline changes in pH resulted in reduced toxin synthesis.

In the present investigation, methods have been developed that allowed the isolation and characterisation of cell-associated α-toxin from Staph. aureus.

MATERIALS AND METHODS

Cultural conditions. An overnight shaken culture of Staph. aureus strain Wood 46 (NCTC 7121) was used to inoculate either six or twelve 2-litre flanged Erlenmeyer flasks each containing 500 ml of a medium free of high molecular weight proteins as described by Bernheimer and Schwartz (1963). The initial pH was adjusted to 7.1 before autoclaving. In some experiments sterile sodium phosphate buffer was added to the medium to a final concentration of 0.1 M. The cultures were grown in an orbital shaking incubator (Gallenkamp, London) at 37°C at a shaking speed of 150 r.p.m. At each time-interval 5- or 10-ml amounts of culture were taken from each flask and bulked. At the end of an experiment no more than 12 per cent. of the culture fluid had been removed from each flask.

Disintegration of cells. Cells were harvested by centrifugation at 17,000g for 10 min. and washed several times with phosphate-buffered saline (PBS) until free from detectable α-toxin activity.

For mechanical disintegration, packed cells from 120 ml of culture were suspended in 20 ml of PBS in a stainless-steel container together with 45 g of no. 10 Ballotini beads; two drops of Silcolapse 5001 antifoam (ICI, Stevenston, Ayrshire) were added to prevent foaming. The cells were shaken intermittently with cooling in the Braun MSK homogeniser at speed-setting 2 for a total of 7 min. After removal of the beads the lysate was centrifuged at 27,000g for 10 min. to remove cell debris.

For enzymic lysis, packed cells from 25 ml of culture were suspended in 2 ml 0.05 M tris (hydroxymethyl)aminomethane-HCl (Tris) buffer at pH 7.5 containing 0.145 M NaCl containing 55 units of lysostaphin (kindly given by Dr P. A. Tavormina, Mead Johnson Research Centre, Evansville, Indiana, USA). After the addition of 0.05 ml saline containing 150 μg of deoxyribonuclease (Seravac Laboratories Ltd, Maidenhead, Berks.) the volume was adjusted to 2.5 ml. The reaction mixture was incubated for 60 min. at 37°C and then centrifuged at 13,000g for 15 min.

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Haemolytic activity. The culture supernatants, pellet "washes", cell lysates and purified fractions were tested for haemolytic activity against rabbit erythrocytes as described previously (McNiven, Owen and Arbuthnott, 1972).

Protein determinations. Protein estimations were carried out by the method of Lowry et al. (1951) with crystalline bovine serum albumin as standard and by ultraviolet extinction measurements at 280 nm in a Hilger-Watts model H700 spectrophotometer with cells of 5-mm light-path.

RESULTS

The levels of extracellular and cell-associated haemolytic activity during growth of Staph. aureus (Wood 46) are shown in fig. 1; in this experiment staphylococcal cells were disintegrated mechanically.

![Graph showing levels of extracellular and cell-associated haemolytic activity during growth of Staph. aureus (Wood 46).](image)

No lag phase was apparent under these growth conditions. Extracellular haemolytic activity, first detected at 2 hr, increased at maximum rate during the logarithmic phase of growth. Haemolytic activity in cell lysates was detected at 5 hr, increased to a maximum at 9 hr as the culture entered the stationary phase, and decreased markedly thereafter.

The level of cell-associated activity was low in comparison with extracellular haemolysin, never exceeding 1 per cent. Although the amounts of extracellular and cell-associated haemolysin varied from experiment to experiment the same over-all pattern was observed consistently. Similar results were obtained by using either mechanical or enzymic disruption. However, the latter method, which was less vigorous, enabled us to detect cell-associated haemolysin at 3 hr.
The decreased synthesis of cell-associated haemolysin after 9 hr is shown in table I, which presents specific activities at different time-intervals during the growth cycle.

**TABLE I**

*Specific activities of cell-associated haemolysin during growth*

<table>
<thead>
<tr>
<th>Sample time (hr)</th>
<th>Specific activity HU per mg protein* rendered detectable by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lysostaphin lysis</td>
</tr>
<tr>
<td>3</td>
<td>2.03</td>
</tr>
<tr>
<td>5</td>
<td>3.44</td>
</tr>
<tr>
<td>7</td>
<td>3.75</td>
</tr>
<tr>
<td>9</td>
<td>4.84</td>
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<tr>
<td>11</td>
<td>...</td>
</tr>
<tr>
<td>12</td>
<td>2.33</td>
</tr>
<tr>
<td>13</td>
<td>2.12</td>
</tr>
</tbody>
</table>

* These results were computed from separate growth experiments.

**TABLE II**

*Purification of cell-associated haemolysin*

<table>
<thead>
<tr>
<th>Stage</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Early stationary phase <em>Staph. aureus</em> (Wood 46) cells washed six times were disrupted mechanically or enzymically</td>
</tr>
<tr>
<td>2</td>
<td>Cell lysate was centrifuged at 39,000g for 20 min.</td>
</tr>
<tr>
<td>3</td>
<td>Supernatant fluid was fractionated by gel filtration on Sephadex G-200</td>
</tr>
<tr>
<td>4</td>
<td>Fractions having maximum haemolytic activity were bulked and concentrated by dialysis against 85 per cent. (w/v) (NH₄)₂SO₄, pH 7.0</td>
</tr>
<tr>
<td>5</td>
<td>Purification by electrofocusing (McNiven et al., 1972)</td>
</tr>
</tbody>
</table>

*Purification and characterisation of cell-associated haemolysin*

In order to characterise this material it was necessary to isolate the relatively small quantity of toxic protein from the mass of cytoplasmic components. The method devised is summarised in table II.

The separation of haemolysin on Sephadex G-200 is shown in fig. 2.

The purified product exhibited the spectrum of haemolytic activity typical of staphylococcal α-toxin. It was neutralised by standard anti α-toxin to the same titre as a corresponding amount of extracellular haemolysin. In electrofocusing studies a single haemolytic component was found (fig. 3). The peak of maximum haemolytic activity (pI 8.79) was one fraction
FIG. 2.—Separation of cell-associated haemolysin on Sephadex G-200. 5 ml lysate containing 65 mg protein applied to a Sephadex G-200 column (27 × 330 mm). The column was eluted with 0.03 M borate buffer pH 8.3. O—O extinction at 280 nm; ▲—▲ haemolytic activity.

FIG. 3.—Electrofocusing of stage 4 cell-associated toxin (the method of McNiven et al., 1972, was used and 24.66 mg of protein were applied to 8101 column) in pH 3–10 gradient. — — pH; O—O extinction at 280 nm; ▲—▲ haemolytic activity.
higher than the corresponding protein peak (pI 8.60). Nevertheless, these values are close to that determined for extracellular toxin (pI = 8.55±0.12) reported earlier (McNiven et al., 1972). Also the molecular weight of 32,500 determined by disk-gel electrophoresis in the presence of sodium dodecyl sulphate is in good agreement with that of the extracellular toxin (McNiven et al., 1972). Therefore purified cell-associated haemolysin closely resembles the extracellular form of staphylococcal α-toxin.

DISCUSSION

The amount of staphylococcal α-toxin associated with cells during growth did not exceed 1 per cent. of the extracellular level. It may be for this reason that Raynaud et al. (1955) failed to detect “intracellular” staphylococcal α-toxin. Although the amount of haemolysin associated with cells is very low, the method described allowed its isolation and characterisation. Using several criteria (haemolysis, neutralisation, iso-electric focusing and molecular weight determination) we established that cell-associated haemolysin closely resembled staphylococcal α-toxin.

With regard to the pattern of production of cell-associated and extracellular α-toxin our findings are in general agreement with those of Hendricks and Altenbern (1968). In both studies the extracellular level of toxin increased throughout the logarithmic phase of growth and remained constant in the stationary phase. The levels of both extracellular and cell-associated α-toxin found in this study were higher than those reported by Hendricks and Altenbern. Also in our study the level of cell-associated toxin reached a maximum at 9 hr (at the onset of stationary phase) in contrast to the peak at 2 hr (mid-logarithmic phase) observed by them. These differences may be due to the use of different strains and growth media. Unlike Hendricks and Altenbern we obtained no conclusive evidence to suggest that the synthesis of toxin in batch culture is controlled by small alkaline changes in pH.

The characteristics of staphylococcal α-toxin production indicate that this toxin is released rapidly after synthesis and unlike certain other bacterial toxins does not accumulate in large amounts within the cell or on the cell surface. We are now investigating the site of α-toxin formation.

SUMMARY

Of the total amount of staphylococcal α-toxin produced in batch cultures, 1 per cent. or less appeared to be cell-associated, indicating rapid release of the toxin into the environment. The level of cell-associated haemolysin reached its maximum at the onset of the stationary phase of growth and decreased thereafter. Cell-associated haemolysin was isolated by the combined use of gel filtration and iso-electric focusing and was characterised by several criteria. Purified material closely resembled the extracellular form of staphylococcal α-toxin.

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REFERENCES


