SHORT COMMUNICATION

A New Haemolysin from Staphylococcus aureus which Lyses Horse Erythrocytes

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A new haemolysin from Staphylococcus aureus produced opaque zones of haemolysis on horse blood agar but did not lyse equine erythrocytes suspended in phosphate-buffered saline. The haemolysin was not neutralized by normal rabbit serum and was distinct from $\alpha$-, $\beta$- and $\delta$-haemolysins as well as human leucocidin. Partially purified preparations produced erythema when injected intradermally into rabbit skin.

INTRODUCTION

The only haemolysin of Staphylococcus aureus which has so far been found to lyse equine erythrocytes is $\delta$-haemolysin (Mollby & Wadstrom, 1973); $\alpha$-haemolysin lysed rabbit and sheep erythrocytes, $\beta$-haemolysin lysed mainly sheep erythrocytes and $\gamma$-haemolysin lysed sheep, rabbit and human erythrocytes.

In assaying $\delta$-haemolysin of S. aureus on horse blood agar, a haemolytic activity was observed that was characterized by an opaque zone of haemolysis that was quite distinct from the clear zones produced by purified $\delta$-haemolysins from S. aureus CN4108 or the canine strain S. aureus CN7450 (Turner, 1978). This paper describes the further characterization of the new haemolysin from a culture supernatant that does not produce $\delta$-haemolysins.

METHODS

Chemicals. Unless otherwise stated, reagents were obtained from BDH and were of Analar grade. Lactic acid was obtained from Sigma, yeast extract and nutrient agar from Oxoid, and Casamino acids and Bacto-agar from Difco. Oxalated horse blood was obtained from Wellcome Research Laboratories.

Strains. Strains of S. aureus were stored on nutrient agar slopes in the dark at room temperature with monthly subculture. Five strains (CN4967, 4967BB, 6708, 6729 and 6730) were isolated from infected rabbits, four (CN7442, 7449, 7450 and 1059) were from dogs, one (CN7451) was of bovine origin and four (Wood 46, Newman D2, Foggie and V8) were of human origin. The canine strain CN 7449 was originally obtained from Dr G. Frazer, Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, Edinburgh.

Growth of bacteria and preparation of crude haemolysin. The strains of S. aureus were cultured in modified CCY medium prepared as described by Arvidson et al. (1971) and were also plated on nutrient agar containing 5% (v/v) sterile oxalated horse blood. Cultures (10 ml) in 100 ml baffled conical flasks were grown overnight at 37 °C on an orbital shaker (140 rev. min$^{-1}$).

Crude culture supernatant containing the new haemolysin was prepared by inoculating 500 ml CCY medium with S. aureus CN7449. The medium contained 30% (v/v) yeast extract diffusate (Gladstone & van Heyningen, 1957) and the cultures were grown in 2 l baffled conical flasks. After 18 h growth at 37 °C on an orbital shaker (140 rev. min$^{-1}$), the culture was centrifuged at 8000 g for 20 min at 40 °C.

Assay of new haemolysin. The new haemolysin was detected on horse blood agar plates which were prepared using a method similar to that reported by Kantor et al. (1972). Indicator agar was made by adding...
Fig. 1. Effect of normal rabbit serum (NRS) and anti-β-haemolysin serum on the zones of lysis formed by the new haemolysin on horse and sheep blood agar: 1, purified δ-haemolysin from S. aureus CN4108 diluted twofold with PBS; 2, purified δ-haemolysin from S. aureus CN4108 diluted twofold with NRS; 3, crude preparation of the new haemolysin from S. aureus CN7449 diluted twofold with PBS; 4 to 7, the same preparation of the new haemolysin as used in 3, in each case diluted twofold with an anti-β-haemolysin serum which was initially undiluted (4), or diluted fivefold (5), 10-fold (6) or 20-fold (7); 8, the same preparation as used in 3, but diluted twofold with NRS; 9, NRS diluted twofold with PBS (to serve as a control for 8); 10, anti-β-haemolysin serum diluted twofold with PBS (to serve as a control for 4, 5, 6 and 7).

1.0 g agar (Difco), 5.84 g NaCl and 10.0 ml 1.0 M-potassium phosphate buffer (pH 7.0) to 75 ml distilled water, heating the mixture to boiling and then cooling to 56 °C. Sufficient distilled water at 56 °C was added until the volume was 90.0 ml followed by 1.0 ml (packed cell volume) washed horse erythrocytes suspended to 10.0 ml in phosphate-buffered saline (PBS, pH 7.0). After thorough mixing 15.0 ml samples were immediately transferred to plastic Petri dishes (8.5 cm diam.). Wells (4.0 mm diam.) were cut in the agar and, after drying for 1 h at 37 °C, the wells were filled with 20 µl of the haemolysin preparation. Plates were examined for zones of haemolysis after 18 h incubation at 37 °C. Human, sheep and rabbit blood agar was made up in the same way using blood to which 0.2 vol. 6% (v/v) sodium citrate in saline had been added.

Purification and assay of δ-haemolysin. δ-Haemolysin was assayed by serially diluting the toxin in PBS in small glass tubes (0.8 x 8.1 cm) and then adding an equal volume of a 1% (v/v) horse blood suspension in PBS. The 50% lysis end-point was determined after 30 min incubation at 37 °C (Turner, 1978). δ-Haemolysin was purified by adsorption on to hydroxylapatite followed by selective desorption (Kreger et al., 1971).

Partial purification of new haemolysin. The new haemolysin was purified by chromatography on a column (2.5 x 40 cm) of Sephacryl S-200. The haemolysin was eluted with PBS (pH 7.2) at 4 °C at a flow rate of approx. 50 ml h⁻¹. Active fractions were shown to be free from Tween 80 lipase (Sierra, 1957), egg yolk lipase (Colbeck, 1956), lysozyme (Jay, 1966), DNAase (Ommen & Friedman, 1970), phosphatase (Cowan & Steel, 1965), caseinase and gelatinase (Cowan & Steel, 1965), α-haemolysin (Adlam et al., 1977) and PV leucocidin (Woodin, 1961). Details of these assays are given in the references cited. β-Haemolysin was assayed by the method of Adlam et al. (1977).

RESULTS

In preliminary experiments, haemolytic zones were obtained with crude culture supernatants on horse, sheep, rabbit and human blood agar. The small, clear zones of haemolysis that were obtained with crude culture supernatant of S. aureus CN7449 on human and rabbit blood agar were replaced by very faint, opaque zones after dialysis of the supernatant against phosphate-buffered saline, whereas the larger opaque zones on horse and sheep blood were unaffected. Purified δ-haemolysin from CN4108 gave a clear zone of lysis on all four types of blood agar.

The zones produced by crude culture supernatants on sheep blood agar were caused by
Fig. 2. Effect produced by the new haemolysin when injected intradermally into rabbit skin: 1, crude preparation of the new haemolysin from *S. aureus* CN7449 after twofold dilution in PBS; 2, the same preparation as used in 1, but dialysed for 18 h against PBS and then diluted twofold in PBS; 3, anti-β-haemolysin serum diluted twofold in PBS; 4, the same preparation as used in 1, but diluted twofold with anti-β-haemolysin serum; 5, the same preparation as used in 2, but diluted twofold with anti-β-haemolysin serum; 6, a sample from a fraction from the preparation of new haemolysin by elution from a Sephacryl S-200 column, diluted twofold with anti-β-haemolysin serum; 7, pooled active fractions of the Sephacryl S-200 preparation diluted twofold with anti-β-haemolysin serum; 8, the same preparation as used in 6, but diluted twofold with PBS instead of anti-β-haemolysin serum; 9, the same preparation as used in 7, diluted twofold with PBS instead of anti-β-haemolysin serum; 10, PBS control. In each case a volume of 0.1 ml was injected.

β-haemolysin since monospecific anti-β-haemolysin serum inhibited zone formation on sheep blood agar but not on horse blood agar (Fig. 1). Although the activity of purified δ-haemolysin towards sheep or horse erythrocytes was totally inhibited by the addition of normal rabbit serum (NRS), activity of the postulated new haemolysin was not affected by NRS.
Attempts to detect haemolysis of horse erythrocytes by the new haemolysin using the procedure normally used for β-haemolysin were invariably unsuccessful. Also no leucocidal activity could be detected against human leucocyte preparations and no clearing of the opaque zones was detected on storing the horse blood agar at 4 °C for 4 to 18 h followed by warming to room temperature (about 20 °C). Crude haemolysin withstood heating to 80 °C for 1 min without loss of activity.

When partially purified haemolysin was injected intradermally into rabbit skin, erythema was produced even in the presence of anti-β-haemolysin serum (Fig. 2). Elution buffer (PBS, pH 7.2) and sterile CCY medium failed to produce erythema.

To investigate how common the new haemolysin was among different strains of S. aureus, 34 strains were inoculated on nutrient agar containing 5% (v/v) horse blood. These strains were also grown in modified CCY medium, and the culture supernatants were examined for the presence of the haemolysin on horse blood agar, both in the presence and absence of NRS. Only two strains showed no lysis when grown on horse blood agar, five strains apparently formed only the new haemolysin and seven strains produced either δ-haemolysin or a mixture of both haemolysins. Crude culture supernatants of the same strains grown in modified CCY medium all contained the new haemolysin activity except two, where large amounts of δ-haemolysin that were not completely neutralized by NRS prevented its identification. It is interesting to note that four strains produced δ-haemolysin in a modified CCY medium but not when grown on horse blood/nutrient agar.

DISCUSSION

The results presented in this paper indicate that S. aureus CN7449 forms a haemolysin which is distinct from δ-haemolysin and produces an opaque zone of haemolysis on horse blood agar plates. This activity was not caused by caseinase, gelatinase, DNAase, phosphatase, Tween 80 lipase, Colbeck egg yolk lipase or lysozyme. The possibility cannot be ruled out that the opaque zones were caused by two or more factors acting synergistically, although, in view of the lack of inhibition of this haemolysin by normal rabbit serum, it is unlikely that δ-haemolysin could be involved in any way. It is perhaps surprising that, of the four species examined, opaque zones were produced only on horse blood agar by the new haemolysin since equine erythrocytes seem to be more resilient than erythrocytes of other species and are not lysed by α-, β- and γ-haemolysins (Mollby & Wadstrom, 1973).

The results obtained by injecting preparations of the new haemolysin intradermally into rabbit skin probably indicate that the haemolysin is responsible for the erythema observed and may indicate that it could play a significant role in the pathogenicity of some staphylococcal diseases. It would be interesting to carry out more studies on this topic using purified material although attempts at purification failed, apparently because the haemolysin was very unstable. As similar haemolytic effects were observed for at least 12 of the 14 strains of S. aureus examined when cultured in modified CCY medium, it seems likely that the haemolysin is common.

A haemolysin designated ζ has been described by Fraser (1964) but this haemolysin produced 'hot/cold' effects on ruminant and rabbit blood as well as wide zones of complete lysis on horse blood agar. Failure to detect any similar effects with the new haemolysin on rabbit and sheep blood agar indicates that the new haemolysin is not related to the ζ-haemolysin which Fraser has described.
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REFERENCES


