IMMUNOGENICITY OF THE δ -HAEMOLYSIN OF *STAPHYLOCOCCUS AUREUS*

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PLATE XIX

ATTEMPTS by several investigators have failed to demonstrate the immunogenicity of the δ -haemolysin of *Staphylococcus aureus*. Gladstone and Yoshida (1967) did not convincingly demonstrate that δ -lysin was immunogenic and they showed that inhibitors of its haemolytic activity were present in serum. Recent evidence suggests that these serum inhibitors are lipoproteins (Donahue, 1969; Kantor, Temples and Shaw, 1972).

In this study, the immunogenicity of δ -lysin has been demonstrated by the use of purified antibody preparations.

MATERIALS AND METHODS

Preparation of δ -lysin

The strain employed, methods of production and purification of δ -lysin were those of Caird and Wiseman (1970). Purified δ -lysin was also kindly sent to us by Dr Arnold Kreger. Haemolysin titrations were performed as described by Wiseman and Caird (1972). Buffers were prepared according to Gomori (1955) and, where required, 0.01M phosphate buffer at pH 7 was supplemented with 0.85% NaCl. Nitrogen content was assayed by the micro-Kjeldahl technique as described by Markham (1942) and an estimate of protein content was derived from the assay value multiplied by 6.25.

Preparation of α -, β - and γ -lysins

α - and γ -Haemolysins were prepared and purified as described elsewhere (Fackrell and Wiseman, 1974; Wiseman, Caird and Fackrell, 1975). Purified β -lysin was used in one immunodiffusion study and was prepared by a modification of the method of Wiseman and Caird (1967) in which the haemolysin was passed through Sephadex G-75 equilibrated with Hallander's (1963) buffer. The active lysin was pooled when removed from the column, dialysed against distilled water for 48 hours, centrifuged and the supernatant fluid lyophilised.

Serological procedures

Immunodiffusion, immunoelectrophoresis and quantitative precipitin methods were those of Campbell *et al.* (1970). The blood agarose required in some immunodiffusion tests was prepared by the addition of 1.0 ml of packed, washed human group-O RBC to 100 ml of a 1% (w/v) solution of Difco agarose in phosphate-buffered saline held at 50°C.

Production of antisera. Antibodies to the haemolysins were prepared in 2-month-old New Zealand white rabbits purchased from the Canadian Research Animal Farm, Bradford,

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Ontario. A solution of purified haemolysin (2 mg/ml) was prepared in phosphate-buffered saline and injected subcutaneously into the animals at 3-day intervals for 3 weeks. After a 2-week period during which no injections were given, the schedule was repeated once. The first three injections of the schedules consisted of 0.1, 0.25 and 0.5 ml of haemolysin followed by 1-ml volumes thereafter. Two days after the last injection, the animals were bled from the heart and the serum was stored at -20°C .

Purification of antisera. Immune and control sera were twice precipitated with ammonium sulphate and fractionated on diethylaminoethyl cellulose (Campbell *et al.*, 1970). The concentration of these purified sera was adjusted to 5 mg protein per ml. A single line of precipitation was observed with goat anti-rabbit serum (Hyland Laboratories, Costa Mesa, California) when the purified antibody was subjected to immunoelectrophoresis.

RESULTS

A single line of precipitation was observed when purified δ -lysin was electrophoresed against purified (IgG) anti- δ -lysin as shown in fig. 1. Ouchterlony agar gel-diffusion studies of the δ -lysin also indicated the presence of one line of precipitation (fig. 2).

FIG. 3.—Quantitative precipitation studies with (left) γ -lysin against purified anti- γ and anti- δ antibody, and (right) δ -lysin against the purified antibodies. ●—● = anti- γ ; ○—○ = anti- δ .

The data obtained in the quantitative precipitin test shown in fig. 3 gave a characteristic plot when increasing amounts of purified δ -lysin were mixed with a constant volume of antibody, yet precipitate was not detected with pre-immune γ -globulin prepared from normal serum. γ -Lysin gave no precipitate with anti- δ -lysin and this proves that the two haemolysins are distinct from each other.

In another experiment, an immunodiffusion test was performed in which the agarose contained human RBC. The slides were developed at 4°C for 2 days and then incubated at 37°C for 1 hour. As shown in fig. 4, the edge of the zone of haemolysis coincided with the precipitin line formed between δ -lysin and its antibody. Pre-immune serum produced no inhibition of haemolysis.

A sample of purified δ -lysin obtained from Dr Kreger (Kreger *et al.*, 1971) gave a line of identity with our δ -lysin when diffused against the antibody (figs. 2 and 4). The Kreger lysin was injected into rabbits and the antiserum obtained was fractionated in the usual manner; this purified anti- δ -lysin gave a

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FIG. 1.—Immunoelectrophoresis of purified δ -haemolysin. The well contains the authors' δ -lysin antigen. The trough contains anti- δ IgG. The anode is to the left. $\times 2.4$.

FIG. 2.—Ouchterlony double diffusion of the authors' purified δ -lysin. Centre well contains authors' δ -lysin. Well (1) contains authors' anti- δ IgG. Well (2) contains IgG prepared from an animal immunised with Kreger's purified δ -lysin. $\times 3.9$.

FIG. 4.—Immunodiffusion of authors' purified δ -lysin in human-blood agarose. Centre well contains δ -lysin. Well (1) contains authors' anti- δ IgG. Well (2) contains authors' pre-immune γ -globulin. Well (3) contains pre-immune γ -globulin from an animal that received Kreger's δ -lysin. Well (4) contains IgG to Kreger's δ -lysin. $\times 2.7$.

FIG. 5.—Ouchterlony double diffusion of Kreger's purified δ -lysin. The centre well contains Kreger's δ -lysin. The outer wells contain the authors' IgG prepared from animals immunised with (1) α -lysin, (2) Kreger's δ -lysin, (3) γ -lysin, and (4) β -lysin. $\times 4.0$.

strong and weak line of precipitation when incubated in an agar gel-diffusion test with Kreger's purified antigen (fig. 5). As shown in the same figure, δ - and γ -lysins were also detected in Kreger's preparation.

DISCUSSION

Unlike Gladstone and Yoshida (1967), we found that in blood-agarose gel-diffusion tests, the line of precipitation resulting from the interaction of δ -lysin with IgG coincided with the zone of haemolysis. Gladstone's inability to observe coincident haemolysin and precipitation zones may have been due to the fact that he was not using a purified antibody. It appears that most of the difficulty in detection of antibody to δ -lysin is due to inhibition of haemolysis by serum proteins other than IgG as shown by Gladstone and Yoshida and others (Donahue, 1969). Kantor *et al.* (1972) have been unable to produce antibody to their δ -lysin preparation and found that lines of precipitation occurred when sera from unimmunised animals were subjected to agar-gel diffusion against the antigen. Whether these animals were unimmunised is open to question and the lines of precipitation they obtained may or may not be regarded as non-specific. It is obvious that reliable results can only be obtained in the presence of purified antibody.

Comparison of Kreger's δ -lysin preparation with our own purified material indicated that the two δ -components are identical antigenically, but it was observed that his lysin also contained α - and γ -lysins. It thus seems that Kreger's method of purification was no more satisfactory than that of Yoshida (1963), whose δ -lysin was subsequently shown to contain β -lysin and ribonuclease. The contamination of Kreger's preparation with α - and γ -lysins might account for the differences between his preparation and our own.

The nature of the curve obtained for δ -lysin in the plot of the quantitative precipitin test data proves that antibody to the lysin was produced. Pre-immune gamma globulin and gamma-lysin IgG were unreactive with the δ -lysin antigen.

SUMMARY

Antibody to δ -lysin of *Staphylococcus aureus* was produced in rabbits given injections of purified antigen. Removal of normal serum inhibitors was effected by ammonium-sulphate fractionation and ion-exchange chromatography. The purified anti- δ -lysin showed a single line of precipitation with purified antigen in Ouchterlony agar gel-diffusion tests and when subjected to immunoelectrophoresis. The line of precipitation also coincided with the zone of haemolysis caused by δ -lysin when the agar gel-diffusion test was performed in blood-agarose plates.

A plot of the data obtained in a quantitative precipitin test performed with purified antibody and δ -lysin showed a characteristic curve confirming their interaction; in control tests with non-immune sera in place of δ -antibody, no precipitation was evident.

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