Immunochemical Studies of *Staphylococcus aureus* Oeding–Haukenes Antigen a₅: a Phosphorus-containing Polysaccharide

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Antigen a₅ was isolated from strain 830 of *Staphylococcus aureus* by autolysis in phosphate buffer followed by alcohol precipitation. Purification was principally achieved by affinity chromatography on wheat germ agglutinin ultrogel and on anti-*S. aureus* teichoic acid immunosorbent. The a₅ antigen was weakly immunogenic in rabbits. Chemical analysis showed that a₅ is a teichoic acid composed of ribitol phosphate, *N*-acetylglucosamine and alanine. It has similar physico-chemical properties to the wall β -*N*-acetylglucosamine ribitol teichoic acid of *S. aureus* but is serologically distinct.

INTRODUCTION

Serological typing of *Staphylococcus aureus* has demonstrated the presence of 30 or more surface agglutinogens or type antigens (Oeding, 1965). Little is known about the chemical nature of these agglutinogens but it is probable that most of them are proteins and the remainder are polysaccharides (Oeding, 1965). Only the n (Groves *et al.*, 1966) and h₁ (Groves *et al.*, 1978) type antigens have been characterized. Oeding (1953, 1954) reported that the agglutinating capacities of staphylococcal polysaccharide antigens in whole bacteria resisted autoclaving and he partially characterized the heat-stable, trypsin-resistant h antigen of strain 17A. This h antigen was later designated a₅ antigen by Haukenes (1964). An epidemiological survey of some wild strains showed that a₅ is a major antigen found in 43% of these strains (Flandrois *et al.*, 1978).

This study was designed to isolate and characterize the a₅ antigen in order to determine its chemical composition and serological reactivity, and to examine the possibility of producing a monospecific antiserum to replace the absorbed whole serum currently used in *S. aureus* serotyping.

METHODS

Strains. *Staphylococcus aureus* strain 830, originally isolated by Haukenes (1963) from a food poisoning case, and obtained from the Czechoslovak Collection of Microorganisms (CCM, Brno, Czechoslovakia), was chosen for the production of the antigen. Other strains, from the same collection, namely 17A, 3647 (which also contain a₅ antigen), 1503, 670, Wood 46, 263 and Cowan I were used for the production of antisera and as controls in serological reactions.

Cultivation. Staphylococci were cultured in 700 ml Todd Hewitt broth (Difco) at 37 °C for 18 h. This starter culture was inoculated into a liquid medium in a 20 litre fermenter (Chemapac, Bron, France), and incubated for

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24 h at 37 °C, pH 7, with forced aeration at an air flow rate of 0.44 l min^{-1} . The medium consisted of 450 g Todd Hewitt broth (Difco), 100 g yeast extract (Difco), 750 g glucose (Merck), 750 g sodium chloride and 15 l distilled water.

The bacterial biomass was collected as a paste after centrifugation at 4000 g and stored at $-18\text{ }^{\circ}\text{C}$. The bacteria used for serological studies were grown in nutrient broth (Oxoid no. 2) containing 50 g NaCl l^{-1} (Fleurette & Modjadedy, 1976).

Extraction of a_5 antigen. A 100 g sample of whole bacteria (wet weight) was suspended in 1 litre 0.066 M-phosphate buffer pH 6.5 and incubated for 24 h at 37 °C, with continuous shaking to promote autolysis. After centrifugation at 7000 g, the sediment was resuspended in the same buffer and extracted as above. The phosphate buffer extraction was repeated three times to remove most of the antigenic material from the cells. All the supernates were pooled.

Preparation of a_5 antigen. Samples were monitored during the preparation by gel immunodiffusion and counter immunoelectrophoresis; the test antisera were anti- α - and anti- β -ribitol teichoic acid (RTA) sera, anti-lipoteichoic acid (LTA) serum, a polyvalent anti-staphylococcal serum raised against strain Cowan I and a_5 -reacting sera (anti-17A, anti-3647 and anti-830). Agglutination inhibition was tested with a specific a_5 serum.

Isolation. Acid precipitation of the pooled supernates was done at 4 °C overnight after adjusting the pH to 4.2 with 1 M-HCl. The suspension was centrifuged at 7000 g for 20 min and the pellet was retained. The supernate was brought to pH 5.2 with 1 M-NaOH and 4 volumes of ethanol were added. Alcohol precipitation was done at $-18\text{ }^{\circ}\text{C}$ for 24 h and the suspension was centrifuged at 7000 g for 20 min. The pellet was dissolved in about 50 ml distilled water.

Purification. The serologically active samples were dialysed against distilled water, lyophilized and redissolved in 10–20 ml distilled water before column chromatography. The material from ethanol precipitation was chromatographed on a column (40 $\times$ 2.8 cm) of DEAE-cellulose (DE52; Whatman) equilibrated with 0.02 M-phosphate buffer pH 7.4. The elution was done with a gradient of 0 to 0.5 M-KCl in 0.02 M-phosphate buffer pH 7.4 at a rate of 30 ml h^{-1} . The serologically active material was applied to a column (1.5 $\times$ 25 cm) of concanavalin A-Sepharose (Pharmacia). The washing buffer was 0.05 M-acetic acid buffer pH 6.0. Elution was done at 15 ml h^{-1} with 0.05 M-boric acid buffer pH 7.8 and 0.05 M-methyl α -D-glucopyranoside dissolved in the washing buffer. The positive fractions were bulked and passed through a column (1.5 $\times$ 15 cm) of wheat germ agglutinin (WGA) ultrogel (IBF, Clichy, France). Washing and elution were done with 0.05 M-Tris/HCl buffer pH 7.8 containing 0.15 M-NaCl and 0.05 M-HCl respectively. The eluted sample was passed through an anti- β -RTA immunosorbent prepared from rabbit anti-teichoic acid IgG as described by Grov (1973) with ACA₄₄ gel (IBF, Clichy, France) activated with cyanogen bromide (Merck). Washing and elution of the column (2.5 $\times$ 20 cm) were done with 0.02 M-phosphate buffer pH 6.8 and 0.01 M-acetic acid buffer pH 3.0 containing 0.2 M-NaCl. The purified product was desalted by dialysis and lyophilized.

Na and K content were monitored by flame photometry and subsequent analyses done only on completely desalted products.

Preparation of β -RTA. The RTA was extracted from strain Wood 46 of *S. aureus* by autolysis and purified by ion-exchange chromatography (Haukenes, 1962). Deacylated LTA was purified from cell autolysates of *S. aureus* Cowan I by ion-exchange chromatography on DEAE-cellulose.

Acid hydrolysis. Samples of 0.5–1 mg were hydrolysed in 0.5 ml 2 M-HCl for 3 h at 100 °C for the analysis of sugars, and in 6 M-HCl for 24 h at 105 °C for amino acid hydrolysis, and evaporated.

Thin-layer chromatography. This was done on silica gel coated glass plates and cellulose plates (Merck) in the following phases: propanol/ammonia/water (6:3:1 by vol.), butanol/acetic acid/water (4:1:1, by vol.), ethyl acetate/pyridine/water (4:1:0.6, by vol.). Alditols were revealed by the periodate/benzidine reagent (Cifonelli & Smith, 1954), sugars and amino sugars by the alkaline silver nitrate reagent (Trevelyan *et al.*, 1950), sugars by the aniline/hydrogen phthalate reagent (Partridge, 1949), and amino acids by reaction with ninhydrin (Consden & Gordon, 1945).

Gas chromatography. Sugars were analysed as their trimethylsilyl alditol derivatives. A reducing mixture (150 μ l) of sodium borohydride (1 mg) and 1 M-ammonium hydroxide (300 μ l) was added at 20 °C to the HCl hydrolysate. After 2 h, 4 ml acetic acid was added and the solvent was evaporated. A 2 ml volume of methanol/HCl (1000:1, v/v) was added and the solvent origin was evaporated. Trimethylsilylimidazole (300 μ l) in pyridine was added and the mixture left to stand for 20 min at 70 °C. A 150 μ l volume of *N*-heptafluorobutyrylimidazole was added and the mixture again left for 20 min at 70 °C. The product was extracted with 250 μ l hexane and 150 μ l water. A 1.5 μ l volume of the hexane layer was injected into a SE 30 wall-coated open tubular glass capillary column (50 m $\times$ 0.35 mm i.d.) (Chrompack, Middelburg, The Netherlands) in a Packard 427 gas chromatograph equipped with a flame ionization detector (Packard Instruments, Delft, The Netherlands). The oven temperature was programmed at $2\text{ }^{\circ}\text{C min}^{-1}$ from 140 °C to 270 °C; the temperature of the injector was 200 °C and that of the detector was 320 °C; quantification was done by using xylose as internal standard.

Amino acids were chromatographed on a Packard 427 gas chromatograph as *N*-heptafluorobutyryl isobutyl ester derivatives (McKenzie & Tenashuk, 1974).

Total phosphorus was determined by the method of Fiske & Subbarow (1925) and hexosamine by a modified Elson-Morgan method (Johnson, 1971).

IR spectroscopy. This analysis was done with a KBr disc. A 1 mg amount of the sample was ground in 250 mg KBr and compressed at a pressure of 10 t in a press (30-ton press C-30; Research and Industrial Instruments Co., London, U.K.) for 20 min *in vacuo* to obtain a clear disc which was then placed in the IR spectrophotometer (model 237 E; Perkin Elmer).

Serological methods. Rabbit antisera were prepared by intravenous injection of formalin-killed bacteria (Oeding, 1957). A sample of *S. aureus* cells (10^9 ml $^{-1}$) was injected three times a week for three weeks. Over this period the following volumes were injected: first week 0.1 ml, 0.1 ml, 0.2 ml; second week 0.2 ml, 0.3 ml, 0.5 ml; third week 0.5 ml, 1 ml, 1 ml. The rabbits were bled 5 d after the last injection. a_5 specific serum was obtained by immunization with strain 830 and absorption with strains 1503 and 670. Strains for absorption were cultivated at 35 °C on nutrient agar for 24 h. The bacterial paste was mixed with the immune serum diluted 1:10 in saline and incubated for 2 h at 35 °C and 18 h at 4 °C. After centrifugation at 3000 g for 15 min the supernate was harvested and tested by seroagglutination of the Oeding-Haukenes set of strains. A specific a_5 serum agglutinates strains 830, 17A and 3647 but not the non- a_5 containing strains like Cowan I, 263 and Wood 46. The amount of absorbing strain is chosen to give absorption of undesirable reactivity of the serum following the scheme of Haukenes (1964). Staphylococcal LTA serum was kindly provided by P. Aasjord, The Gade Institute, Bergen, Norway.

Immunization with a_5 was done by intradermal inoculation of rabbits with 0.5 mg a_5 in Freund's complete adjuvant (Bio Merieux, Marcy l'Etoile, France). A booster inoculation was given six weeks later by intramuscular injection of 0.2 mg a_5 in incomplete adjuvant (Bio Merieux). Blood samples were monitored each week for antibodies by immunodiffusion and agglutination tests. The rabbits were bled five weeks after the booster injection.

Inhibition of agglutination. (See Flandrois *et al.*, 1981.) The specific a_5 serum was titrated by serial dilutions and the highest dilution of serum which gave detectable agglutination of the test strains 830 and 17A was the agglutinating titre. Serum was used for inhibition of agglutination tests at half this titre so that, mixed with an equal volume of a solution containing an unknown amount of a_5 , the serum retained its agglutinating titre only if no a_5 was present. The inhibition tests were done at 20 °C for 2 h with regular agitation. Presence of a_5 was shown by inhibition of agglutination in these conditions due to lowering of the serum agglutinating titre.

Immunodiffusion was done in a 10% (w/v) agar medium containing 8.5% (w/v) NaCl and 0.005% (w/v) NaN₃, buffered at pH 7.4 with 0.1 M-barbital buffer. A Gelman immunoplate device (Gelman Instrument Co., Ann Arbor, Mich., U.S.A.) was used for all immunodiffusion procedures. Wells 5 mm apart were filled with undiluted rabbit sera or with the antigen extracts, and the plates were left for 18 h at 24 °C. The plates were stained with amido black after extensive elution of free protein from the agar with saline and water (Flandrois *et al.*, 1975).

Immunoelectrophoresis and counter immunoelectrophoresis were done on glass plates (10 × 10 cm) coated with 1% (w/v) agarose (IBF, Clichy, France) on 0.1 M-barbital buffer pH 8.6 for 30 min at 10 V cm $^{-1}$. Counter immunoelectrophoresis detected 1.0 µg α - or β -RTA with antisera to either 263 or Wood 46.

RESULTS

Most of the a_5 antigenic material detected by agglutination inhibition and gel immunodiffusion was precipitated with alcohol. The acid precipitate contained little a_5 material and was insoluble in water. The elution profile is shown in Fig. 1. Fraction I contained both a_5 and RTA. Fraction II has been found serologically and chemically similar to deacylated LTA. The material excluded from concanavalin A-Sepharose (Fig. 2), was shown by agglutination inhibition and immunodiffusion to consist of a_5 and β -RTA. Neither α -RTA nor any other antigen was detected by counter-current immunoelectrophoresis or by gel immunodiffusion against anti-263 and anti-Cowan I serum. Use of WGA-Sepharose gave disappointing results since WGA fixed both a_5 and RTA, the only difference being that a higher amount of a_5 fixed on the lectin. Despite several attempts, we failed to separate the two antigens on WGA-Sepharose.

Separation was successful (Fig. 3) on the anti- β -RTA immunosorbent column, and no better method was found for separation. The degree of separation was shown by counter immunoelectrophoresis of the eluate against anti- β -RTA serum (Wood 46 antiserum). Even at a concentration of 10 g l $^{-1}$ the final material tested by counter immunoelectrophoresis was devoid of any reactivity against anti- α -RTA, anti- β -RTA, anti-LTA and anti-Cowan I sera. After 6 h a single immunodiffusion arc was formed against anti-830 or anti-17 serum. At a lower concentration (0.25 g l $^{-1}$) purified a_5 inhibited the agglutination of a_5 -containing strains in a_5 -specific serum but inhibited no other agglutination reaction in the Oeding-Haukenes system.

Fig. 1. Fractionation of ethanol-precipitated antigenic material on DEAE-cellulose. The column was equilibrated in 0.2 M-phosphate buffer pH 7.4. Elution was at a rate of 30 ml h^{-1} with a linear gradient of 0 to 0.5 M-KCl in 0.02 M-phosphate buffer pH 7.4. Fractions of 10 ml were collected. Peaks A and B were UV-absorbing substances (proteins and nucleic acids). Fraction I consisted of a_5 and wall RTA of *S. aureus*. Fraction II contained the deacylated LTA of *S. aureus*.

Fig. 2. Affinity chromatography of a_5 and RTA on a concanavalin A-Sepharose column at a rate of 15 ml h^{-1} . Washing buffer, A, 0.05 M-acetic pH 6.0. Elution with B, 0.05 M-boric acid buffer pH 7.8 followed by C, 0.05 M-methyl α -D-glucopyranoside in 0.05 M-acetic acid buffer pH 6.0. Fractions of 5 ml were collected.

Fig. 3. Fractionation of partially purified a_5 on an anti β -RTA immunosorbent column. Washing and elution were with 0.02 M-phosphate buffer pH 6.8 (A) and 0.01 M-acetic acid buffer pH 3 containing 0.2 M-NaCl (B). Flow rate 15 ml h^{-1} . Fractions of 3 ml were collected.

Fig. 4. Immunoelectrophoresis of purified a_5 antigen and β -RTA (1 mg ml^{-1}). Anti- a_5 serum was antiserum to strain 17A of *S. aureus*; this serum gave no reaction against α - and β -RTA. Anti- β -RTA serum was antiserum to strain Wood 46 of *S. aureus*.

The final product at a concentration of 5 g l^{-1} showed no UV-absorption in the range 250–300 nm.

Figure 4 illustrates the absence of reactivity by immunoelectrophoresis between a_5 and an anti- β -RTA (Wood 46) serum. The same absence of precipitation was observed against anti-263 serum which detected both α - and β -RTA. No precipitate formed between pure a_5 and a_5 specific serum, probably because of the low titre of the specific serum. Double diffusion in agar gel showed an absence of cross-reactivity between a_5 and β -RTA with no reaction of identity between the two arcs.

Immunization of rabbits with the a_5 antigen gave specific a_5 antisera. The rise in antibodies was obtained 5–7 weeks after the booster injection. A maximum agglutination titre of 800 was obtained against strains 830, 17A and 3647, and no reaction was observed with non- a_5 -containing strains (titre of 100 or less). This serum reacted in gel immunodiffusion with purified a_5 but not with α - or β -RTA and deacylated LTA. The agar precipitation line was weak and only apparent with undiluted serum.

The amount of final material obtained was 8 mg from 100 g of bacteria (wet weight). Thin-layer chromatography of 2 M-HCl hydrolysates of a_5 antigen revealed the major components as ribitol, anhydroribitol, ribitol phosphate, glucosamine and *N*-acetylglucosamine. Alanine was the main amino acid detected from the 6 M-HCl hydrolysate. The same components were obtained from β -RTA under similar conditions.

The molar proportions of phosphorus, glucosamine, ribitol and alanine in a_5 antigen were respectively 1:0.8:0.64:0.22. Trace amounts of glycine, glutamic acid and lysine were obtained from chromatograms of *N*-heptafluorobutyryl isobutyl esters. This derivative also gave a good separation of anhydroribitol, ribitol and glucosamine, thus allowing a comparative study of the products. The ratio of anhydroribitol to ribitol in a_5 antigen was less than that of β -RTA (0.05 and 0.15 respectively).

The IR spectra of a_5 antigen and β -RTA were identical with characteristic bands at 1550 cm^{-1} and 1650 cm^{-1} , corresponding to δ amide and ν (C=O) vibrations of the *N*-acetyl group. This confirms the *N*-acetylation of glucosamine.

DISCUSSION

An appreciable amount of a_5 antigenic material was obtained from whole-cell autolysates of strain 830. This antigenic material was heavily contaminated by the wall RTA of *S. aureus*. An attempt to obtain a strain possessing a_5 without the RTA proved futile since most strains contain RTA detectable on whole bacteria or in extracts of the strains. This contamination by RTA necessitated a rather rigorous purification process. This problem was partially resolved by the use of affinity chromatography on WGA-Sepharose. The anti- β -RTA immunosorbent column enabled us to complete purification of the a_5 antigen as demonstrated by the absence of β -RTA in the excluded effluents (a_5 fractions) of this column in counter immunoelectrophoresis (less than 1% contamination by α - and β -RTA or LTA). The detection methods used did not permit the quantification of the yield during the extraction and for each purification step. However, the final amount of pure material was about 8 mg a_5 from 100 g (wet weight) bacteria. This is far lower than the usual β -RTA of *S. aureus*, which represents 30–40% of the cell wall (Sanderson *et al.*, 1962). During this extraction process the yield of the usual β -RTA was about ten times that of the a_5 obtained; a_5 is therefore a minor component of the wall.

The presence of ribitol, anhydriitol, ribitol phosphate, *N*-acetylglucosamine and alanine indicates that a_5 is a ribitol-type teichoic acid (Baddiley, 1970; Knox & Wicken, 1973). The small amount of purified material obtained did not permit further elaboration of its structure.

The chemical nature of a_5 is consistent with some of its previously known properties: thermostability and resistance to trypsin (Oeding, 1955). Like other non-protein antigens, its production is not hindered by the salt content of the growth medium (Haukenes, 1964).

Gel chromatography and chemical analyses show that a_5 has the same physical and chemical properties as β -RTA. The lack of reactivity between a_5 antigen and concanavalin A-Sepharose gel demonstrates that a_5 antigen contains neither a terminal α -linked *N*-acetylglucosamine nor an α -glucoside (Reeder & Ekstedt, 1961). It is also possible that the amounts present were insufficient for demonstrable reactivity (Sanderson *et al.*, 1961). On the other hand, the affinity of a_5 for WGA shows that the *N*-acetylglucosamine may be β -linked; but the interpretation of WGA affinity is difficult as the affinity varied with the molarity of the eluting buffer (Ndulue *et al.*, 1981). Antigen a_5 is not retained on anti- β -RTA columns and there is no reaction of identity between a_5 and α - or β -RTA. This was expected because previous work showed that the a_5 antiserum agglutinated strains containing a_5 antigen after absorption with reference strains possessing β -RTA (Haukenes, 1964; Fleurette & Modjadedy, 1976).

The complete immunologic unrelatedness of a_5 and α - and β -RTA demonstrated in all reactions employed is, however, difficult to explain. This could be due to either (a) *N*-acetylglucosamine occupying a position in the chain different from the position of this amino sugar in the conventional β -RTA of *S. aureus*, or (b) the presence of a peptidoglycan fragment structurally distinct from the one attached to β -RTA. Although the presence of only traces of glycine, glutamic acid and lysine is more in accordance with their presence as contaminants than with a role in immunological reactivity, this could not be proved from our data. Furthermore, alanine, the only amino acid present in high amounts, could be linked to the sugar chain in a way different from that in β -RTA to explain the immunological difference.

The absence of precipitate between a_5 and its specific serum is certainly explained by the low titre of specific sera produced from rabbits immunized by whole cells, as shown by Oeding and Haukenes (Oeding, 1965).

Evidence of a unique defined antigen corresponding to the specific serum a_5 used for *S. aureus* serotyping is shown by the complete absence of agglutination of a_5 -containing strains in a purified a_5 -absorbed serum. Evidence of a unique defined structure corresponding to a_5 specific serum used in serotyping confirms the earlier findings of Haukenes (1964), who differentiated the a_5 antigen from other staphylococcal antigens without extraction by cross-absorption studies and agglutination. Antigen a_5 is the third purified antigen among the Oeding-Haukenes type antigens and, like the two others, it corresponds to a definite antigenic entity. These results therefore support the Oeding-Haukenes serotyping of *S. aureus*. Antigen a_5 is the only demonstrated *S. aureus* type antigen of teichoic acid nature, the other known antigens being

proteins or glycopeptides (Grov *et al.*, 1966, 1978). This will lead to a possible classification of Oeding-Haukenes type antigens on an immunochemical basis.

Burger (1966) and Haukenes (1962) did not obtain anti-teichoic acid antibodies with acid-extracted teichoic acid. In accordance with the results of Oeding (1955), the a_5 antigen was weakly immunogenic. The high agglutination titre obtained as compared with the very low precipitation titre (whole serum only gave a weak line) could be explained by the presence of IgM antibodies (Pike, 1967). Yoshida & Ekstedt (1968) also obtained IgM anti-teichoic acid antibodies on immunization of rabbits with *S. aureus*. The use of a_5 -RTA for the production of a serotyping factor serum is therefore limited by the relatively low titre of the immune sera obtained and the difficulties involved in getting a high yield of antigen free from the usual β -RTA.

Immunochemical study of *S. aureus* agglutinogens seems promising for the recognition of new structures associated with the bacterial surface, for chemical classification of Oeding-Haukenes antigens and, when immunization problems are solved, for the production of antisera.

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