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Preparation of Cell Wall Antigens of *Staphylococcus aureus*

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Cell walls were prepared from *Staphylococcus aureus* strains Copenhagen and 263 by high-speed mixing in the presence of glass beads followed by differential centrifugation. Insoluble peptidoglycan complexes were derived from cell walls by extraction of teichoic acid with 10% trichloroacetic acid. Intact teichoic acid was prepared from each strain by digestion of cell walls with lysostaphin and isolated by column chromatography. Soluble glycopeptide (peptidoglycan in which only the glycan has been fragmented) and the stable complex of teichoic acid with glycopeptide were prepared by digestion of cell walls with *Chalaropsis* B endo-*N*-acetylmuramidase and were separated by column chromatography. Amino acid and amino sugar contents of walls and subunits of walls were comparable to those reported by others.

Recent studies on pathogenesis and immunity in experimental model infections with *Staphylococcus aureus* have been directed to the roles in these processes of surface antigens (13), cell walls and the wall teichoic acid (2), and mural peptidoglycans (10, 11). Although there is evidence that hypersensitivity to staphylococcal cells is significant in pathogenesis (12), the antigens involved have not been defined.

The cell walls of staphylococci consist mainly of peptidoglycan and teichoic acid polymers and are very resistant to common hydrolytic enzymes. In fact, no known enzyme is able to hydrolyze teichoic acid, the peptide of the peptidoglycan is totally resistant to mammalian peptidases, and lysozyme can hydrolyze the glycan slowly and only after removal of teichoic acid. No other mammalian enzyme is able to solubilize the peptidoglycans of bacterial cell walls, and many intact walls, such as those of *S. aureus*, are almost totally resistant to lysozyme. Thus, these polymers could be expected to contribute to the intraphagocytic survival of staphylococci (7).

Both the teichoic acids (22) and the polypeptide of the mural peptidoglycan (11) have been shown to be determinants for antibody production, but their role in hypersensitivity of the delayed type has not been explored. In addition to these components, the cell walls of *S. aureus* contain other components, including protein antigens (8), autolytic enzymes (18), and phage receptor sites (1).

The peptidoglycan and teichoic acid components of staphylococcal cell walls can be obtained free from these other components in soluble form by a variety of enzymatic and chemical procedures, and the purpose of our investigations was to evaluate some of their immunobiological characteristics. The preparation of the antigens is reported here.

MATERIALS AND METHODS

Organisms. Two strains of *S. aureus* were used as the source of antigens. Strain 263 was obtained from J. Cohen, Communicable Disease Center, Atlanta, Ga., and strain Copenhagen was from the culture collection of the Department of Pharmacology, University of Wisconsin Medical School.

Frozen stock cultures were prepared from each of the strains after the selection of isolated colonies from sheep blood-agar plates. These cultures were passaged twice in 9.5 ml of Trypticase soy broth (TSB) prior to inoculation of 100 ml of TSB in 250-ml flasks. After incubation at 37 C for 16 hr on a rotary shaker, the cells were collected by centrifugation at 11,000 × *g* for 15 min. After two distilled-water washings, a 10% (w/v) suspension was prepared, distributed in 75.0-ml quantities, and stored at −10 C.

Preparation of cell walls. Cell walls were prepared by high-speed mixing with glass beads and differential centrifugation (6). The contents of a sterile, precooled 200-ml Sorval Omni-mixer homogenizer vessel, immersed in an ice-salt bath and containing 75 ml of 10% cell suspension, 75 ml of glass beads, and 0.25 ml of tri-*n*-butyl phosphate, were intermittently mixed at full speed for 12 to 16 min. The crude cell walls were recovered by decantation and washing the beads with distilled water. The walls were collected by centrifugation at 14,500 × *g* for 10 min, followed by two washings with distilled water, three washings with

1.0 M NaCl, and three washings with potassium phosphate buffer (*p*H 7.0). Additional purification was accomplished by six cycles of differential centrifugation at 750 × *g* for 5 min followed by 14,500 × *g* for 10 min. All of these procedures were performed at 0 to 5 C, and cell walls were then immersed in a boiling-water bath for 20 min to inactivate autolytic enzymes.

Most crude cell wall preparations were further purified by incubation at 37 C for 2 hr with 200 μg of ribonuclease per ml (Calbiochem, 5× crystalline) in 0.1 M potassium phosphate buffer (*p*H 7.0). Trypsin (Nutritional Biochemicals Corp., 2× crystalline) was then added, and incubation continued for an additional 4 hr. After enzyme digestion and 6 to 10 washings with distilled water, the purified cell walls were frozen and dried in vacuo over NaOH pellets.

Cell wall preparations were monitored by electron microscopy, after staining with 2% phosphotungstic acid.

Preparation of trichloroacetic acid-extracted cell walls [peptidoglycan (PG) complex]. Cell walls were extracted with 10% trichloroacetic acid at 60 C for 3 hr (3). The PG complex was collected by centrifugation, washed with distilled water, frozen and dried in vacuo over NaOH pellets.

Digestion of strain 263 cell walls with Chalaropsis B endo-N-acetylmuramidase. Approximately 500 mg of purified 263 cell walls was suspended in 0.01 M sodium acetate buffer (*p*H 4.5) with 1 mg of *Chalaropsis* B enzyme (gift of J. Hash, Vanderbilt University School of Medicine, Nashville, Tenn.) at 37 C for 18 hr. At intervals through 18 hr, samples (10 μliter) were removed and frozen. Reducing power was measured on 1-μliter portions of the samples. Similar preparations had been made previously from Copenhagen cell walls (20).

Digestion of cell walls with lysostaphin. A 200-mg amount of cell walls of each strain was incubated at 37 C with 2 mg of lysostaphin (gift of P. Tavormina, Mead Johnson and Company, Evansville, Ind.) in 0.05 M potassium phosphate buffer (*p*H 7.5). After 12 hr, an additional 2 mg of enzyme was added and incubation continued through 42 hr. At intervals, samples (10 μliter) were removed, added to 100 μliters of distilled water, and boiled for 2 min. A 10-μliter portion of each of the diluted samples was analyzed for reducing power.

Ion-exchange chromatography and gel filtration. Enzyme digests were fractionated on a column (2 by 35 cm) of ECTEOLA-cellulose (Cellex E, BioRad Laboratories, Richmond, Calif.) which had been previously washed with 0.5 M LiCl and water. Gel filtration was carried out on a column (2 by 50 cm) of Bio Gel P-2 which had been washed with deionized water. All columns were operated at room temperature, and water was used as the initial eluant for all columns (18).

Analytical procedures. For the determination of amino acids and amino sugars, 1.0-mg samples of the various antigens were hydrolyzed in 200 ml of 6 N HCl at 105 C in sealed tubes for about 18 hr. After hydrolysis, the samples were diluted with 415 μliters of citrate buffer (*p*H 2.2), and samples were applied to the columns of a Beckman-Spinco amino acid analyzer (18).

Reducing power was determined by a micromodification of the ferricyanide method (5). The method of Lowry et al. (15) was employed for determination of phosphate.

Immunological methods. Rabbits were immunized by repeated intravenous inoculations of washed Formalin-treated whole cells (17). Sera were analyzed for precipitating antibodies in Ouchterlony plates with wells 6 mm in diameter, placed 15 mm apart. Sera were placed in the center wells and allowed to diffuse for 24 hr before the six outer wells were charged with antigen (9). The plates were held at 4 C in a moist chamber and examined daily.

RESULTS

The various batches of cell walls prepared were generally comparable in amino acid and amino sugar composition and morphology in electron micrographs to those prepared by other workers (14, 16, 21).

The amino acid and amino sugar compositions of representative individual batches of 263 and Copenhagen cell walls are presented in Table 1. With the exception of glycine and alanine, the values for which were lower than expected, the molar ratios of each of the other constituents, per mole of glutamic acid, compared favorably with the reported values (21). The molar yields of individual constituents, per gram of 263 cell walls, were also lower than expected, indicating the presence of considerable amounts of material giving no color with ninhydrin after hydrolysis. Electron micrographs of this preparation of 263 cell walls revealed the presence of an electron-dense material (Fig. 1).

Analyses of trichloroacetic acid-extracted cell walls. Amino acid and amino sugar analyses of PG complexes from each of the strains are shown in Table 2. Since pure *S. aureus* peptidoglycan has a repeating disaccharide-nonapeptide subunit

TABLE 1. *Analyses of cell walls of strains 263 and Copenhagen*

Amino acids and amino sugars	263		Copenhagen	
	Moles[a]	Ratio	Moles	Ratio
Glutamic acid	0.299	1.00	0.461	1.00
Lysine	0.323	1.08	0.468	1.01
Alanine	0.662	2.21	0.975	2.11
Glycine	1.247	4.17	2.803	3.91
Muramic acid	0.282	0.94	0.446	0.97
Glucosamine	0.712	2.38	1.223	2.65
Ammonia	0.334	1.12	0.473	1.03

[a] Moles per gram of cell walls. Only trace amounts of other amino acids were present.

having a molecular weight of approximately 1,200 (21), the Copenhagen preparation has the expected glutamic acid content, whereas the 263 preparation still gives evidence of impurity. Ratios of lysine, alanine, muramic acid, and glucosamine per mole of glutamic acid compared favorably with reported values (14, 16, 21). Values of glycine and ammonia were lower than expected. In these preparations, approximately 8 to 10% of the total phosphate of the cell walls remained with the PG complex and could not be removed by prolonged extraction with trichloroacetic acid. A comparison of analytical data for cell walls and PG complexes (Tables 1 and 2) indicates that extraction with trichloroacetic acid did not result in selective solubilization of PG components, and recovery of PG was quantitative.

TABLE 2. *Analyses of peptidoglycan complexes of strains 263 and Copenhagen*

Amino acids and amino sugars	263		Copenhagen	
	Moles[a]	Ratio	Moles	Ratio
Glutamic acid	0.680	1.00	0.811	1.00
Lysine	0.713	1.05	0.823	1.01
Alanine	1.597	2.35	1.748	2.15
Glycine	2.863	4.21	3.354	4.13
Muramic acid	0.669	0.98	0.848	1.05
Glucosamine	NA[b]		1.016	1.25
Ammonia	0.611	0.90	0.500	0.62

[a] Moles per gram of peptidoglycan complex.
[b] Not available.

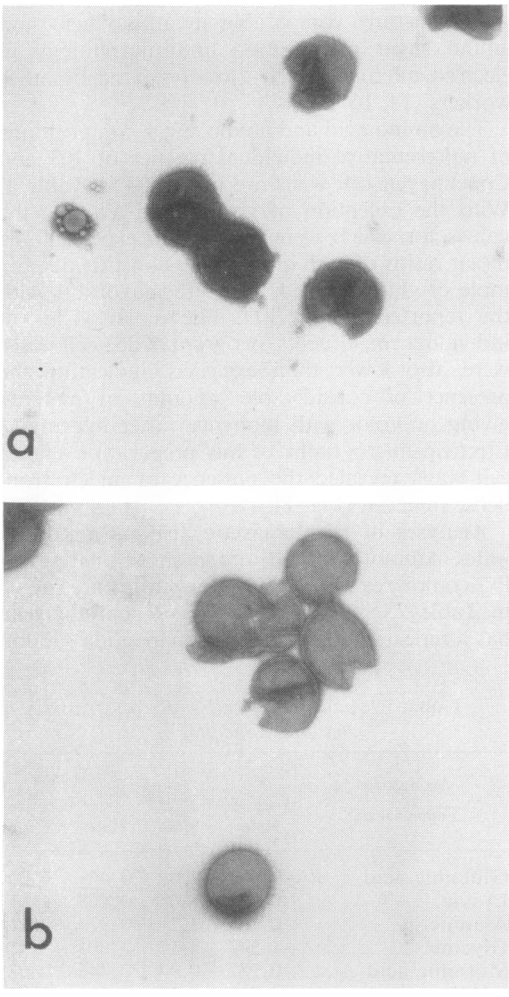

FIG. 1. *Electron micrographs of staphylococcal cell wall preparations stained with 2% phosphotungstic acid. (a) Strain 263 cell walls, (b) Copenhagen cell walls.* × *10,000*

Fractionation of Chalaropsis B digest. Release of reducing power was completed in 2 hr and approximated that for release of 1 mole of 4-O-β-*N*-acetylglucosaminyl-*N*-acetyl muramic acid disaccharide per mole of total glutamic acid (5). Reducing power did not increase after addition of more *Chalaropsis* B enzyme. After 18 hr, the digest was clarified by centrifugation at 15,000 × *g* for 20 min. The supernatant was decanted, and the pellets of nonpeptidoglycan material were washed with a small volume of distilled water. The supernatant and wash were applied to a column of ECTEOLA-cellulose, followed by a small volume of deionized water.

Fractions eluted with water which contained measurable reducing power were pooled and lyophilized. The material in this peak has been identified as the soluble glycopeptide (PG in which only the glycan has been fragmented) in which 4-O-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides are attached by *N*-acetylmuramyl-L-alanine linkages to the intact polypeptide, which retains its native random degree of polymerization (18). This product should thus have many of the antigenic properties of the peptide of the peptidoglycan.

A second peak of reducing power was eluted with 0.7 M LiCl. The contents of tubes with reducing power were pooled, concentrated by flash evaporation, and extracted with 95% ethyl alcohol to remove LiCl. Desalinization was completed by filtration on a column of Bio-Gel P-2. Similar antigens had been prepared previously from strain Copenhagen (18). This fraction of the *Chalaropsis* B digest has been characterized as a stable complex of teichoic acid covalently linked with glycopeptide fragments of random chain length (18) in which the teichoic acid retains its

TABLE 3. *Analyses of principal amino acids and amino sugars of enzymatically solubilized teichoic acid of strain 263*

Amino acids and amino sugars	Strain 263	
	Moles[a]	Ratio
Glutamic acid	0.025	1.00
Lysine	0.028	1.10
Alanine	0.068	2.72
Glycine	0.069	2.74
Muramic acid	0.109	4.35
Glucosamine	0.911	36.07
Ammonia	0.132	5.26
Phosphate	1.822	72.20

[a] Moles per gram of teichoic acid.

ester-linked D-alanine residues. It is designated TAPG fragments.

Fractionation of lysostaphin digests. Lysostaphin solubilizes *S. aureus* cell walls by endopeptidase action on their pentaglycine crossbridges from which neutral tri- and tetraglycine peptides are excised. Subsequent action of *N*-acetylmuramidase-L-alanine amidase results in liberation of hexosamine-free basic peptide fragments and intact glycan. The endo-*N*-acetylglucosaminidase of lysostaphin is able to hydrolyze the glycan only after it has been stripped of its peptide by the action of the endopeptidase and amidase components of the mixture (19). Release of reducing power thus demonstrates completion of all of these steps and complete fragmentation of the peptidoglycan. The teichoic acid is left with a minimal fragment of glycopeptide attached, and the material should have the antigenic properties of native teichoic acid except for the loss of most of its ester-linked D-alanine residues. The products of enzymatic hydrolysis can be fractionated by ion-exchange chromatography.

Release of reducing power proceeded slowly, so digestion with lysostaphin was continued for 42 hr. The digests were immersed in boiling water for 2 min and then clarified by centrifugation at 14,000 × *g* for 10 min. The supernatants were decanted, diluted to 20 ml with deionized water, and fractionated on a column of ECTEOLA-cellulose (18). The fraction eluted with water, which contained a peptide and oligosaccharides, was discarded without analysis. A gradient of linearly increasing LiCl concentrations up to 0.7 M was then applied. All tubes which gave a positive test for chloride were assayed for total phosphate and inorganic phosphate.

The first phosphate peak, composed entirely of inorganic phosphate, was derived from the buffer of the digestion mixture and was discarded. The second broader peak was devoid of inorganic phosphate. It was pooled, concentrated, and repeatedly extracted with 95% ethyl alcohol until the extracts were free from chloride. The final precipitate was dissolved in deionized water, frozen, and dried in vacuo over NaOH pellets.

On the basis of amino acid, amino sugar, and phosphate analyses of 263 teichoic acid (Table 3), it was concluded that it contained less than 1% of associated peptidoglycan fragments and little if any ester D-alanine. The phosphate to glucosamine ratio also indicated that the ribitol residues of this teichoic acid were not fully substituted with glucosamine.

Immunodiffusion test. Solutions (1 mg/ml) of 263 teichoic acid, 263 TAPG fragments, Copenhagen teichoic acid, and Copenhagen TAPG fragments were examined with homologous and heterologous antisera. Results with 263 antiserum are shown in Fig. 2, taken at 48 hr. Precipitation was observed first at 30 hr with homologous teichoic acid. Precipitin arcs, which gave reactions of identity with 263 teichoic acid, then developed in order with 263 TAPG fragments, Co-

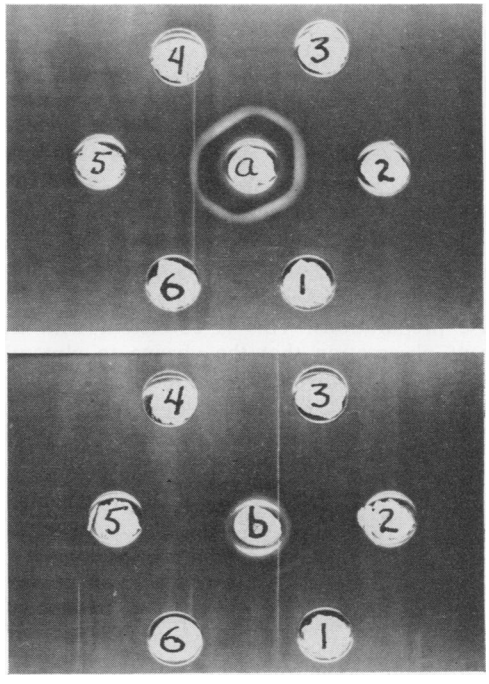

FIG. 2. *Immunodiffusion patterns of staphylococcal cell wall antigens. Central wells contained anti-263 serum (a) and anti-Copenhagen serum (b). Peripheral wells contained solutions (1 mg/ml) of Copenhagen teichoic acid (1), 263 teichoic acid (2), 263 TAPG (3), Copenhagen TAPG (4), 263 teichoic acid (5 and 6).*

penhagen teichoic acid, and Copenhagen TAPG fragments. Copenhagen antiserum precipitated only with homogous teichoic acid (Fig. 2).

DISCUSSION

The qualitative amino acid and amino sugar compositions of the cell wall from *S. aureus* strains 263 and Copenhagen are similar, and their molar ratios are generally comparable to those prepared by other workers. The particular preparations of the two strains in Fig. 2 were made for a specific animal experiment which is to be described in a subsequent paper. Amino acid and amino sugar analyses shown in Tables 1 and 2 were also from these preparations and from the PG complexes derived from them. In electron micrographs of this preparation of 263 cell walls, an electron-dense substance was seen associated with the walls. Amino acid analyses corroborated the presence of a contaminating substance as evidenced by the yields of individual cell wall constituents per gram of cell wall. This material cannot contain protein because analyses did not reveal amino acids other than those generally accepted as cell wall components. Since the total phosphate values were not elevated, it cannot be nucleic acid and is probably inorganic.

Although the molar ratios of some individual cell wall constituents were lower than the reported values, some of these, such as ester-linked alanine of teichoic acid or the amino sugars, are labile structures.

The molar ratio of glycine to glutamic acid in cell walls and PG complexes of either strain was 10 to 20% lower than the previously reported ratio of 5:1. Because of the consistency of this observation, it appears to be a characteristic of these preparations. This is consistent with the recent observation that the glycine to glutamic acid ratio of *S. aureus* wall peptidoglycan appears to depend upon the amino acid composition of the medium in which the cells are grown and is only maximally five (O. Kandler, *personal communication*).

The molar ratios for alanine were also low in the cell walls but not in the PG complexes, indicating that the teichoic acids were not fully substituted with alanine, some of which is known to be hydrolyzed by boiling of walls in distilled water (D. J. Tipper, *unpublished data*).

Enzymatic, nonautolytic methods were selected for the preparation of cell wall antigens, because the hydrolytic specificity of the enzymes was known. As a result, the structure of the hydrolysis products could be reliably predicted. Teichoic acid produced by enzymatic means has a higher molecular weight that that extracted with trichloroacetic acid (4), thus making it more suitable as a skin testing and precipitating antigen.

Although the number of sera examined by immunodiffusion was limited, the analyses suggest that the sera had antibody specific for teichoic acid determinants. This is based on the fact that the first precipitate to appear with anti-263 serum was with its homologous teichoic acid. Subsequently, reactions of identity with homologous and heterologous antigens appeared. In addition, the only reaction observed with anti-Copenhagen serum was with its homologous teichoic acid. The immunodiffusion patterns also indicate that the anti-263 serum had antibody for a determinant common to both strains, whereas the anti-Copenhagen serum reacted only with the homologous determinants.

It has been shown (22) that two lines are obtained in immunodiffusion with teichoic acid and human sera only when the teichoic acid contains close to equal proportions of α- and β-linked *N*-acetylglucosamine. Failure of the Copenhagen antiserum to precipitate with 263 teichoic acid antigens suggests that most of the antibody in this serum is directed against β-N-acetylglucosamine, since the 263 contains mostly the α-linked polymer. Confirmation of this would require quantitative hapten-inhibition studies.

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