

Filamentous Capsulated Streptococci from the Human Respiratory Tract

II. Antigenic Structure of Provisional Capsular Types 89 and 83/89

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Two immunologically distinct polysaccharides have been isolated from the filamentous alpha-hemolytic streptococcus of provisional capsular type 89, recovered from the human respiratory tract. Chemical analyses indicate that the capsular polysaccharide consists of glucose, galactose, and a small amount of rhamnose, whereas the cell wall-associated polymer contains galactosamine, glucosamine, glucose, and phosphorus. Immunological studies suggest that the capsular polysaccharide is type specific and that the cell wall-associated carbohydrate, which cross-reacts with the C₈₉, or cell wall-like capsular polysaccharide of pneumococcus, may be group specific. A noncapsulated variant of the prototypic streptococcus of provisional type 89 was shown to possess the same cell wall-associated carbohydrate as the strain from which it was derived, but it proved to be poorly antigenic in rabbits. A filamentous capsulated streptococcus reacting with antisera to filamentous streptococci of both provisional capsular types 83 and 89 has been found to produce two capsular polysaccharides, each of which reacts with antibody to one of the aforementioned untypic strains and represents an unusual binary capsulated streptococcus.

It has been found previously that many strains of alpha- and nonhemolytic streptococci isolated from human respiratory secretions produce type-specific capsular substances (2, 11). Extensive immunological studies of a collection of filamentous morphological variants giving rise to rugose colonies on the surface of agar plates have revealed the existence of at least 30 capsular serotypes. In many instances, strains of these filamentous capsulated streptococci have also been shown to produce a cell wall antigen related immunochemically to the C, or cell wall, polysaccharide of pneumococcus (2). A recent investigation of the prototypic strain of the filamentous alpha-hemolytic streptococcus of provisional capsular type 83 has shown that it possesses a cell wall antigen consisting of galactosamine, glucosamine, glucose, and phosphorus as well as capsular antigen composed of galactose and phosphate (10). Because of the potential importance of being able to demonstrate the presence of a common cell wall antigen which might serve as a basis for grouping filamentous streptococci of diverse capsular types, additional data were sought. In the present report, the cell wall and capsular antigens were isolated from the prototypic strains of

the filamentous streptococci of provisional capsular types 89 and 83/89. The purification and immunochemical characterization of these antigens are described, and evidence is presented which indicates that the streptococcal strain of provisional type 83/89 is a naturally occurring binary capsulated streptococcus.

MATERIALS AND METHODS

Bacterial strains. The prototypic strains of filamentous alpha-hemolytic streptococci with provisional capsular designations of types 83, 89, and 83/89, referred to hereafter as types 83, 89, and 83/89, respectively, were used. These strains were isolated from respiratory secretions of patients at the Philadelphia General Hospital, but bore no necessary relationship to the illnesses for which examination of sputum was requested. Streptococcal strain 89R50 is a noncapsulated mutant derived from the streptococcal strain of provisional type 89 after 50 serial daily cultures in broth containing 20% antiserum to the capsular antigen.

Pneumococcal strain R36NC is a noncapsulated variant of the capsulated type II strain, D39S, carried in the laboratory for many years (3).

Preparation of bacterial carbohydrates. Carbohydrates were extracted from intact streptococcal cells with cold trichloroacetic acid by the method of Park and Hancock (15).

Chromatography. DEAE-cellulose chromatography was performed by a method described previously (12), as was Bio-gel filtration (9). Paper chromatography was carried out by the ascending technique of Pazur et al. (16).

Analytical methods. Analytical procedures for rhamnose, glucose, glucosamine, galactose, galactosamine, amino acids, and phosphorus have been described previously (5-7, 12, 17, 19).

Immunologic methods. Quantitative and qualitative precipitin analyses, double diffusion in agar gels, immunoelectrophoresis, and preparation of antisera in rabbits were performed by methods described previously (10, 14, 18).

RESULTS

Isolation of polysaccharides from filamentous type 89. Whole cells of filamentous streptococcus type 89 were extracted with cold trichloroacetic acid by the method of Park and Hancock (15), and the extract was dialyzed, concentrated, and deproteinized. Analysis of the crude trichloroacetic acid extract with the capillary precipitin technique indicated that it contained at least two distinct polymers: an antigen reacting with an antiserum to streptococcus type 89, and an antigen reacting with an antiserum to the C_6 , or cell wall-like capsular polysaccharide of pneumococcus (4). In view of this observation, attempts were made to separate the two antigens by diethylaminoethyl (DEAE)-cellulose chromatography (12). Illustrated in Fig. 1 is the pattern of the trichloroacetic acid extract eluted from a DEAE-cellulose column (30 by 2.5 cm) with increasing

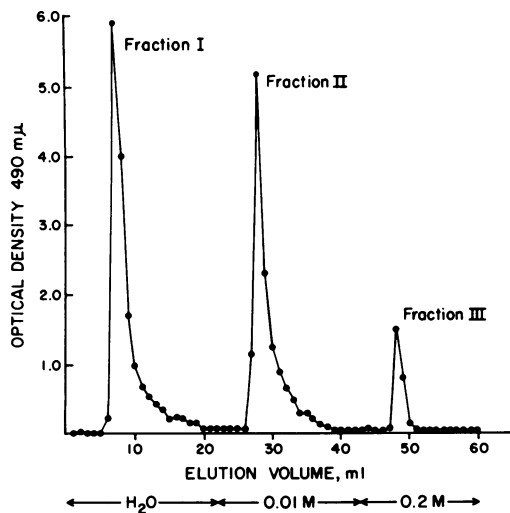


FIG. 1. DEAE-cellulose column chromatography of a 50-mg sample of a trichloroacetic acid extract of whole cells of streptococcus type 89. Column was 30 by 2.5 cm. Buffer: $(NH_4)_2CO_3$, pH 8.6, at 4 C. Eluted material was pooled into fractions I, II, and III as designated in the figure.

concentrations of $(NH_4)_2CO_3$. Three major peaks were present in the eluate from the ion-exchange column which were designated fractions I, II, and III, respectively. Capillary precipitin tests indicated that fractions I and II represented antigens with the capsular specificity of type 89, whereas fraction III represented an acidic polymer which cross-reacted with antiserum to the C_6 polysaccharide of pneumococcus. Ascending paper chromatography analysis of acid hydrolysates of fractions I and III substantiated the view that the two antigens are immunochemically distinct polymers. Fraction I consisted of approximately equimolar concentrations of glucose and galactose with minimal amounts of rhamnose. In contrast, fraction III consisted of glucosamine, galactosamine, and glucose. Fractions I and III were further purified by P-30 Bio-gel filtration and analyzed chemically (Table 1). Note that the crude trichloroacetic acid extract (column 1) contains significant amounts of glucose, galactose, and phosphorus. Purified fraction I consists of equimoles of glucose and galactose and a small amount of rhamnose. In contrast, fraction III consists of high levels of glucose and phosphorus and moderate levels of hexosamines and galactose. The results of the chemical analysis of the two fractions, together with those of capillary precipitin tests, indicate that fraction I represents the type-specific capsular antigen and fraction III the cell wall antigen of streptococcus type 89.

Immunologic analysis of the capsular antigen of streptococcus type 89. Illustrated in Fig. 2 are the results of quantitative precipitin tests performed with the type 89 streptococcal capsular antigen and homologous and heterologous

TABLE 1. Chemical composition of a crude trichloroacetic acid extract of streptococcus type 89 and of two antigenic fractions isolated therefrom

Component	Crude 89 trichloroacetic acid extract ($\mu\text{mol}/\text{mg}$)	Fraction I ($\mu\text{mol}/\text{mg}$)	Fraction III ($\mu\text{mol}/\text{mg}$)
Glucose	1.43	2.25	1.44
Galactose	0.96	2.28	0.28
Rhamnose	0.53	0.24	0.01
Phosphorus	1.22	<0.01	2.23
Glucosamine	0.36	<0.01	0.54
Galactosamine	0.30	<0.01	0.18
Lysine	0.05	<0.01	ND ^a
Glutamic acid	0.05	<0.01	ND
Glycine	0.08	<0.01	ND
Alanine	0.10	<0.01	ND
Muramic acid	<0.01	<0.01	ND

^a ND, not done.

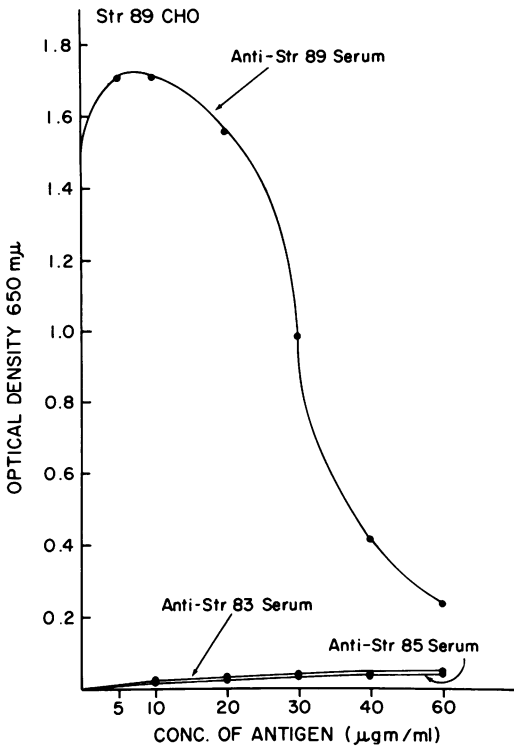


FIG. 2. Quantitative precipitin reactions between purified type 89 capsular polysaccharide and 0.1 ml of antisera to streptococcal types 83, 85, and 89, respectively.

antisera. The purified type 89 capsular antigen (fraction I) gave a strong precipitin reaction with homologous antiserum. On the other hand, this same antigen failed to react with antisera to capsulated streptococci of either provisional types 83 or 85. These findings are clearly compatible with the view that the capsular antigen of type 89 is, in fact, type specific.

Immunology of the cell wall antigen of streptococcus type 89. Initial results indicated that the cell wall antigen of streptococcus type 89 can be isolated readily from whole bacterial cells by the sequential procedures of extraction with cold trichloroacetic acid (15) and of DEAE-cellulose chromatography (12) (Fig. 1). In most instances, however, the yield of cell wall material was small, and it was frequently contaminated with the type-specific capsular antigen. Because of this difficulty, a noncapsulated mutant of type 89 was isolated after passage of the strain in broth containing 20% homologous anti-capsular serum for 50 consecutive daily transfers. The noncapsulated mutant, designated strain 89R50, was grown in Todd-Hewitt broth, and the cells were extracted with cold trichloro-

acetic acid. Paper chromatography analysis of the preparations of cell wall antigens derived from the noncapsulated mutant strain 89R50 and from the parental strain capsulated type 89 indicated that the two cell wall antigens are chemically analogous. In both, the major constituents were glucose, galactosamine, and glucosamine. Double diffusion studies in agar demonstrated that the cell wall antigen of the noncapsulated mutant strain was immunologically identical to the antigen derived from the parent strain. The precipitin lines formed between the cell wall antigen of each strain and antiserum to pneumococcal C_s polysaccharide merged to form a line of identity.

Structural analysis of the capsular antigen of streptococcus type 89. The capsular antigen was subjected to hydrolysis in 0.1 N HCl at 100 C for varying periods of time, and the hydrolytic products were analyzed by the paper chromatography methods described by Pazur et al. (16). Analysis of the acid hydrolysate of the type 89 capsular antigen obtained after 5 min revealed only glucose. Hydrolysates obtained after longer exposure to acid revealed the presence, in addition to glucose, of galactose as well as a series of oligosaccharides in the di-, tri-, and tetrasaccharide range. These findings suggested that the type 89 capsular antigen may consist of a linear polymer with branches composed of glucose residues at the nonreducing terminals. To obtain further information about the structural features of the type 89 capsular antigen, oligosaccharides were isolated from the partial acid hydrolysate of the polysaccharide by the method of multiple ascent paper chromatography (9, 16), and their structures were analyzed. The oligosaccharides were designated A1, A2, B, and C, respectively. Fragments A1 and A2 had *R_f* values which appeared to correspond to those of disaccharides, fragment B to that of a trisaccharide, and fragment C to that of a tetrasaccharide. Fragment A1 had an *R_f* value similar to that of lactose, a β-1-4-D-galactosyl-*o*-glucose; the *R_f* value of fragment A2 was similar to that of allolactose, a β-1-6-D-galactosyl-*o*-glucose. In both instances, the hydrolysates of fragments A1 and A2 revealed the presence of glucose and galactose after treatment with beta-glucosidase. Alpha-glucosidase was inactive against both of these fragments. The results suggest that fragments A1 and A2 are disaccharides consisting of beta-linked galactosyl-glucose.

Oligosaccharides isolated from the partial acid hydrolysate of the type 89 streptococcal capsular antigen were employed subsequently as potential inhibitors in the quantitative pre-

cipitin inhibition test. Attempts to obtain information on the antigenic determinant of the type 89 capsular antigen with the use of glucose and galactose as well as of fragments A1, A2, B, and C in this test gave equivocal results. The failure of the oligosaccharides to inhibit the precipitin reaction between the type 89 capsular antigen and its homologous antiserum could be attributable to the possibility that the oligosaccharides isolated are structural components of the carbohydrate backbone rather than components which comprise the immunodominant terminal residues. In view of this possibility, attempts were made to remove selectively terminal monosaccharide residues with specific glycosidases. Previous experiments had indicated that glucose residues might occupy a terminal position on the side chains of the type 89 capsular polysaccharide. The purified antigen was treated β - and α -D-glucosidases, and the enzymatic hydrolysates were analyzed by paper chromatography. These experiments demonstrated that the capsular antigen was resistant to the action of both enzymes. In addition, the antigen was resistant also to the action of β -D-galactosidase. These results suggest either that glucose is not the terminal residue of the polymer or that the terminal glucose residues may contain substituent groups, such as acetyl groups, which render them more resistant to the action of specific enzymes.

Immunochemistry of the capsular antigens of streptococcus type 83/89. A strain of alpha-hemolytic filamentous streptococci which gave positive capsular precipitin (Quellung) reactions with antisera to streptococci of both provisional capsular types 83 and 89 was examined to determine whether its capsule was composed of a single polysaccharide reacting with each antiserum or if it was a binary capsulated strain producing two distinct surface antigens. Illustrated in Fig. 3 are the results of the immunoelectrophoretic analysis of a trichloroacetic acid extract of this organism with homologous antiserum. The findings indicate that the extract (well 1) contains two distinct antigens, a neutral and an acidic polymer. Note that the acidic polymer in the extract of type 83/89 has an electrophoretic mobility similar to that of the purified capsular polysaccharide of type 83 (well 2). Additional electrophoretic study of the extract indicated that the neutral polymer observed in Fig. 3 is immunologically analogous to the purified capsular antigen of type 89.

The two distinct antigens in the extract of type 83/89 were separated by DEAE-cellulose chromatography as previously described (12). Two major fractions were recovered from the

column, one eluted with water (fraction I), the other eluted with 0.1 M $(\text{NH}_4)_2\text{CO}_3$ (fraction II). Chemical analysis of fraction I showed it to contain approximately 28% glucose, 21% galactose, and 5% rhamnose, whereas fraction II contained 13% glucose, 24% galactose, and 9% phosphorus. The chemical analyses indicate that fraction I represents the neutral type 89 capsular polysaccharide and that fraction II, although possibly contaminated with elements of cell wall antigen, represents the acidic type 83 capsular antigen.

Illustrated in Fig. 4 are the results of quantitative precipitin tests with the two fractions isolated from the extract of type 83/89 and antisera to streptococci of types 83 and 89. Fraction I gave a strong precipitin reaction with the antiserum to type 89 and only a minimal reaction to type 83. In contrast, fraction II gave a strong precipitin reaction with antiserum to type 83 but did not react to type 89. The following double diffusion experiment (Fig. 5) underscores the immunochemical identity of the two capsular antigens of type 83/89 with the purified capsular antigens of types 83 and 89 in their reactions with antiserum to type 83/89. It can be seen that the capsular antigens of type 83/89 (well 1) form two distinct precipitin bands with antiserum to the homologous organism. The outer precipitin band merges with that formed between the purified capsular antigen of type 89 and the same serum to form a line of identity. In analogous fashion, the inner precipitin band merges with that formed between the purified capsular antigen of type 83 and antiserum to type 83/89. The results demonstrate clearly that the filamentous alpha-hemolytic

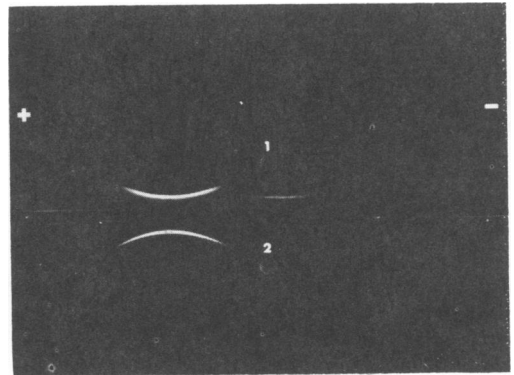


FIG. 3. Immunoelectrophoresis of a trichloroacetic acid extract of whole cells of streptococcus type 83/89. Well 1, crude trichloroacetic acid extract of whole cells of type 83/89; well 2, purified capsular polysaccharide of type 83; trough, antiserum to type 83/89. Concentration of antigens was 0.1 mg/ml.

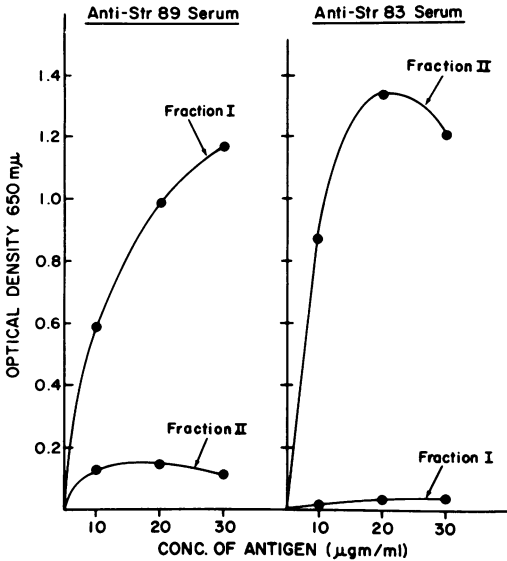


FIG. 4. Quantitative precipitin reactions. Left frame: Quantitative precipitin reactions between fractions I (type 89 capsular component) and II (type 83 capsular component) isolated from streptococcus type 83/89 and 0.1 ml of antiserum to type 89. Right frame: Quantitative precipitin reactions between the same fractions isolated from type 83/89 and 0.1 ml of antiserum to type 83.

streptococcus of provisional capsular type 83/89 is a binary capsulated organism producing two distinct capsular polysaccharides, each of which appears identical with the capsular antigen of another previously identified untypic strain of filamentous streptococci.

DISCUSSION

Previous studies have indicated that many strains of alpha- and nonhemolytic filamentous streptococci inhabiting the human respiratory tract may be classified serologically on the basis of their type-specific polysaccharides (2). In addition, a number of strains of diverse capsular types possess cell wall-associated antigens which are related immunologically to the C and C_s polysaccharides of pneumococcus, a finding which suggests that they may form a distinct serogroup (2, 11). In an extension of these studies, the cellular antigens of two related filamentous streptococcal strains of provisional capsular types 89 and 83/89 were isolated, purified, and characterized immunochemically. The results obtained give added support to the feasibility of classifying filamentous streptococci of the human respiratory tract into distinct groups on the basis of the antigenic heteropolymers of their cell walls. Two immunologically active carbohydrates were iso-

lated from the cells of type 89, a capsular polysaccharide, and a cell wall polysaccharide which was shown to be analogous to the cell wall antigen of type 83 (11). In addition, the cell wall antigens of both streptococcal types were found to be related immunochemically to the C_s or cell wall-like capsular polysaccharide of pneumococcus (4).

Chemical analyses of the capsular polysaccharide of type 89 indicate that it consists of equimoles of glucose and galactose and small amount of rhamnose. Structural information derived from identification of hydrolytic fragments of the type 89 capsular antigen and from immunological studies, however, have not revealed the immunochemical basis for its antigenic specificity. After complete acid hydrolysis, the capsular antigen was converted to glucose and galactose in equimolar concentrations. On graded acid hydrolysis, glucose appeared as the initial product of low molecular weight, whereas galactose and low-molecular-weight oligosaccharides were released subsequently. The disaccharides from the antigen have been tentatively identified as lactose and allolactose. It should be emphasized that all of the oligosaccharides studied were inactive as inhibitors of the reaction between the type 89 capsular polysaccharide and its homologous antiserum. The findings indicate that these oligosaccharides are not essential components of the immunodominant determinant of the

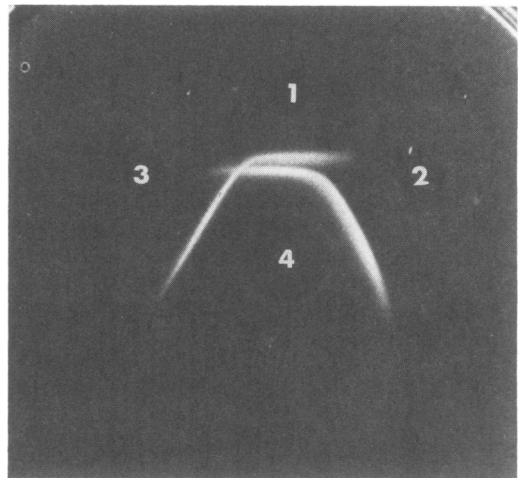


FIG. 5. Immunodiffusion reactions in agar of the purified capsular polysaccharides of streptococcal types 83 and 89 and a crude preparation of antigens of type 83/89 with antiserum to type 83/89. Well 1, crude antigens of type 83/89; well 2, purified type 83 capsular polysaccharide; well 3, purified type 89 capsular polysaccharide; well 4, antiserum to type 83/89. Concentration of antigens was 0.1 mg/ml.

capsular polysaccharide and perhaps represent components of its backbone structure. The inability to remove glucose as well as galactose from the polymer by enzymatic hydrolysis together with the ineffectiveness of these hexoses as inhibitors of the precipitin reaction suggest that the immunodominant determinant of the type 89 capsular polysaccharide may be a monosaccharide with substituent groups or a disaccharide of unusual structure.

The filamentous streptococcus of provisional type 83/89 would appear to be an unusual organism. It had been shown previously by the Quellung technique that it gave positive reactions with antisera to the capsular polysaccharides of streptococci of both provisional types 83 and 89. In accord with these findings, two capsular polysaccharides were isolated from a trichloroacetic acid extract of whole cells of type 83/89 by DEAE-cellulose chromatography. Immunological studies demonstrated that the acidic antigen, a galactose-rich polymer, reacted with antiserum to type 83, whereas the neutral antigen, a glucose-galactose polymer, reacted with antiserum to type 89. The finding of binary capsulation in a wild-type streptococcus would appear to be an unusual one. Although simultaneous production of two capsular polysaccharides by pneumococcus after transformation has been demonstrated (1), no naturally occurring strain of this organism has been shown to have this attribute. Production of two exocellular polysaccharides by capsulated strains of *Streptococcus salivarius* grown on sucrose is known (8), but the levan formed from sucrose does not form a discrete capsular component.

In addition to the two capsular polysaccharides of streptococcus type 83/89, a cell wall-associated polymer was isolated from the prototypic strain. This cell wall polysaccharide, like those from the prototypic strains of provisional types 83 and 89, cross-reacts with the C₆ polysaccharide of pneumococcus. This observation enhances the likelihood that a number of strains of filamentous streptococci of diverse capsular types, isolated from the human respiratory tract, may possess the same or immunologically similar cell wall polysaccharides. If this inference proves to be the case, these cell wall antigens could form the basis for the grouping of strains of diverse capsular serotypes in a manner analogous to that employed in the classification of the several groups of beta-hemolytic streptococci. It is noteworthy that considerable difficulty has been encountered in selecting noncapsulated mutants of filamentous streptococci by passage of strains in medium contain-

ing homologous capsular antibody. The autoagglutinability of these strains probably lessens the selective nutritional advantage of such noncapsulated variants that may arise by mutation in the medium employed, as does the failure of cells in long filaments or chains to separate from one another after division. Although a noncapsulated variant, strain 89R50, was recovered from the prototypic filamentous streptococcus of provisional capsular type 89, vaccines of this variant have proved to be weakly antigenic in rabbits, and other mutants are being sought. The availability of antibody of high titer to streptococcal cell wall polysaccharides would aid significantly efforts to group these organisms serologically.

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