CELL-WALL COMPOSITION AND THE GROUPING ANTIGENS OF STREPTOCOCCI

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ABSTRACT

SLADE, HUTTON D. (Northwestern University Medical School, Chicago, Ill.) AND WILLIAM C. SLAMP. Cell-wall composition and grouping antigens of streptococci. J. Bacteriol. **84**:345–351. 1962.—The carbohydrates present in the cell walls of streptococci belonging to serological groups A–H and K–S, and unclassifiable strains, have been identified. The sugars found were rhamnose, glucose, galactose, arabinose, and mannose. All sugars vary considerably in their distribution among the groups; glucose, galactose, and rhamnose occur most frequently. Strains were found which contained each of the latter sugars singly or in combination with one or both of the other sugars. Variation within a single group occurred in one-half of the groups. A strain containing only glucose and another only galactose were found. Except for groups A and C, in which only rhamnose is present in the great majority of strains, the presence or absence of the sugars does not aid in the identification of the groups. The cell walls of all groups examined also contained alanine, glutamic acid, lysine, glucosamine, galactosamine, and muramic acid. The cell walls of all groups, except D, agglutinated in the presence of specific group antisera, indicating the presence of the group antigen in the cell wall. Strains in groups F, K, and M gave a weak reaction. The structure and chemical composition of the group antigens of the streptococci are discussed.

Little information is available on the composition of the cell walls of the 17 serological groups of streptococci. The bacterial cell wall, and surface structures in particular, are important as regards the antigenicity and pathogenicity of many bacterial species. It appeared worthwhile to examine the walls of the streptococci for the presence of compounds which would illustrate differences among the groups and thus serve as a basis for future investigations. The carbohydrates were selected for this purpose. It was also deemed essential to examine a sufficient number of strains in each serological group so that a reliable expression of the characteristics of each would be obtained. Evidence for the presence of additional substances in the cell walls will be presented, as well as information on the location of the group antigen in the streptococcal cell. Preliminary reports have been published (Slade, 1961*a*, *b*).

MATERIALS AND METHODS

Cultures. Strains were received from the following: National Collection of Type Cultures, American Type Culture Collection, R. Lancefield, W. Maxted, R. M. Cole, M. McCarty, A. Bahn, R. Pakula, and from the collection of E. Todd. We express our appreciation for the many cultures received.

Serological procedures. The serological grouping reaction of the strains was determined by the precipitin test (Swift, Wilson, and Lancefield, 1943) with rabbit antisera received from the Communicable Disease Center, U.S. Public Health Service, Atlanta, Ga. Strains belonging to P, R, and S were checked by W. R. Maxted. In some cases, antisera were prepared in rabbits in our laboratory. As a general procedure, the first two injections (0.5 ml each) were followed by eight injections (1 ml each) of a formalin-killed suspension of 17-hr cells grown in Todd-Hewitt broth (Difco). Injections were spaced 2 to 3 days apart, and bleeding was performed approximately 1 week after the last injection. Suspensions contained 10 mg (dry wt) of cells/ml.

A second procedure was also employed in the preparation of the suspensions for injection. Lyophilized cells were suspended (10 mg/ml) in 0.9% saline and shaken for 10 min with Ballotini beads in a Mickle shaker. A ratio of 5 ml of suspension to 5 g of beads was employed. The beads were removed on a coarse glass filter, and formalin was added to 0.05%. The injec-

tion schedule was similar to that employed with whole cells.

The agglutination method of Cummins (1954) was used to detect the presence of the group-specific antigen in the cell wall. Lyophilized cell walls, as prepared above, were shaken again with glass beads (10 mg of cell walls; 5 ml of water; 2 g of beads) for 5 min, and made to 10 ml. Two drops of 12% sodium azide were added as a preservative. (The authors are indebted to Dr. Cummins for the preparation of some of the cell-wall suspensions and determination of the agglutination titer.)

Growth of streptococci. Organisms were grown in quantities of 1 to 2 liters for 18 hr in Todd-Hewitt broth plus a glucose-salts mixture (Hess and Slade, 1955). The procedure for harvesting of cells and preparation of cell walls has been reported (Slade and Slamp, 1960).

Chromatographic procedures. Approximately 30 mg of lyophilized cell walls were hydrolyzed in 4 ml of 2 N H_2SO_4 in sealed vials at 100 to 105 C for 2 hr, neutralized with $Ba(OH)_2$, filtered, dried under vacuum at room temperature, and dissolved in 0.3 ml of water; 125 μliters of hydrolyzate representing about 12 mg of cell walls, were applied to each paper sheet. Two-dimensional chromatography on Whatman 3 MM paper was employed. The first solvent (80% phenol) was run on the long axis of the paper, followed by lutidine (65%) on the short axis. The carbohydrates were spotted by dipping the paper in aniline phthalate (aniline, 2 ml; phthalic acid, 3.3 g; acetone, 95 ml; water, 5 ml) and heating at 110 C for 12 min. A standard made up of glucose, galactose, arabinose, mannose, and rhamnose was used as a check. In case of doubt as to the identity of a spot, a known sugar or sugars was added to the hydrolyzate and repeat chromatograms were run. The relative quantity of each carbohydrate is expressed as 4+, 3+, 2+, +, and trace (tr) depending upon the size and intensity of the spot. Recent quantitative data indicate that trace quantities represent approximately one-fifth of those sugars present in maximal amount.

For the detection of amino acids, amino sugars, and muramic acid, the same conditions were employed except that phenol was run on the short axis of the paper, and lutidine on the long axis. The compounds were detected by dipping in 0.2% ninhydrin in 95% acetone-5% water, and heating for 3 min at 110 C.

RESULTS

Each strain examined in all of the known serological groups of streptococci, with the exception of groups O, K, and R, contained rhamnose in the cell wall (Table 1). In group O, no strains positive for rhamnose were found, whereas three of six strains in group K, and one of two strains in R did not contain this methyl pentose. Among the unclassifiable strains, two of five lacked rhamnose. The single O strain examined by Roberts and Stewart (1961) likewise lacked rhamnose. The absence of rhamnose in a significant proportion of the group K and unclassifiable strains in the present study is in agreement with their work.

Table 1 shows that glucose was the second carbohydrate most frequently found in the streptococcal cell wall. It was uniformly absent in group A, except for a small quantity in strain AH1 and K43v, and in group C. Its presence was variable in groups G and L and in the unclassifiable group. Strain 734 in group R contained only glucose.

Roberts and Stewart (1961) reported the presence of both glucose and galactose in strain AH1 (group A) but upon further examination were not able to verify the original finding of galactose (Stewart, *personal communication*). The latter finding is in agreement with the present results.

Galactose was found less frequently than either rhamnose or glucose. It was uniformly absent in groups A, C, and E, and variable in groups D, F, H, R, and S. A single strain of all those examined (K61, unclassifiable group) contained only galactose. Strain MD, in the same group, contained only a trace of glucose in addition to galactose. All strains in B, M, N, P, and Q contained glucose, galactose, and rhamnose.

Arabinose has been found for the first time in streptococci, in two of five strains in the serologically unclassifiable group, and in one of six strains in group K. The quantity of arabinose was appreciable, especially in strain 6249 (Table 1).

Mannose has been found for the first time in strains belonging to group K and the unclassifiable group. In agreement with Cummins and Harris (1956), strain 5385 of group E also contained mannose; however, in contrast, we have not found strain 370 (group D) to contain this carbohydrate.

The optical rotation of glucose in nine strains belonging to groups F, K, M, N, O, S, and the unclassifiable group was examined with D-glucose

TABLE 1. *Carbohydrates in cell walls of streptococci*

Serological group	Strains	Rhamnose	Glucose	Galactose
A	TIM, C203, C121, T3M, Blackmore, 553, Kilborne	4+	–	–
	AH1, K43v	4+	+	–
B	Brownette, 10790, Lewis, 090R	4+	2+	2+
C	26RP66	4+	tr	–
	H861, K2033	4+	–	–
D	7379, 8175, 8176, 8177, 8213, 8307, DH	3+	4+	2+
	370	3+	4+	–
E	K129, 5385[a]	4+	3+	–
F	C628, DS1340	4+	4+	2+
	H60R	4+	4+	–
G	F68A, Valenti	4+	2+	+
	8539	4+	tr	tr
	9603	4+	–	3+
H	Hockley, F90A, Sbe	3+	3+	+
	7865, 7868	4+	4+	–
	Challis, 9124	4+	+	–
K	3472, D34E, Sal[b]	3+	4+	2+
	D34B, GD, Murphy	–	2+	4+
L	DS4880, DS5440	3+	4+	3+
	D167A	4+	–	3+
M	54X136, 54X134	4+	2+	3+
N	C559, B210, B207	4+	3+	2+
O	B357, B361, 4835, 1360	–	3+	4+
P	9824, 54X853	3+	3+	2+
Q	E1551, E6844, E6849, R6566	3+	2+	4+
R	734	–	2+	–
	735	4+	2+	3+
S	1545	tr	4+	tr
	428	2+	4+	–
	1424	2+	3+	2+
	Unclassifiable			
	6249[c], 9811M78[d]	+	3+	tr
	MD	–	tr	4+
	K61	–	–	2+
	903	2+	3+	3+

[a] Contains mannose (+).
[b] Contains mannose (tr), arabinose (tr).
[c] Contains mannose (+), arabinose (2+).
[d] Contains mannose (tr), arabinose (+).

oxidase (Glucostat, Worthington Biochemical Corp.). The D form was found in each case. The rotation possessed by rhamnose, galactose, arabinose, and mannose must await the examination of larger quantities of cell wall. Rhamnose in one strain from group A is known to be the L form (Barkulis and Jones, 1957).

Table 2 lists the amino acids, amino sugars, and muramic acid present in the cell walls of strains from groups B, G, H, K, L, M, N, O, P, Q, S, and the unclassified group. In each case, significant quantities of alanine, glutamic acid, lysine, and muramic acid were present. These results indicate that the mucopeptide found in other gram-positive bacteria (Perkins and Rogers, 1959; Strominger, 1960) occurs also among the various serological groups of streptococci. The presence of glucosamine and galactosamine in each strain is likewise shown in Table 2. These amino sugars most likely exist in the cell wall as the *N*-acetyl derivatives; however, during acid hydrolysis the acetyl group is removed.

TABLE 2. *Amino acids and amino sugars in cell walls of streptococci*[a]

Group	Strains	Amino acids				Amino sugars		Muramic acid
		Alanine	Glutamic acid	Lysine	Aspartic acid	Glucosamine	Galactosamine	
B	Brownette	4+	3+	2+	+	4+	3+	3+
G	F68A	4+	2+	+	−	2+	+	3+
H	F90A, Challis	4+	3+	2+	+	+	4+	4+
K	3472, D34B	4+	2+	2+	−	3+	3+	4+
L	DS4880	4+	2+	+	−	2+	4+	3+
	D167A	4+	2+	+	−	2+	+	3+
M	54X134	4+	3+	2+	+	3+	−	4+
N	C559	4+	2+	tr	+	2+	4+	4+
O	B361, 1360	4+	4+	2+	−	3+	+	4+
P	9824	4+	4+	3+	+	3+	−	4+
Q	E1551	4+	3+	+	2+	2+	+	4+
S	1424	4+	3+	2+	+	2+	tr	4+
UN[b]	K61	4+	3+	2+	+	2+	tr	4+

[a] The intensity of the amino acid and amino sugar spots should be considered separately. Alanine, for example, possessed a greater intensity in all cases than any of the amino sugars.

[b] Serologically unclassifiable strain.

In groups A (Schmidt, 1952) and C (Krause and McCarty, 1962), the specific carbohydrate antigen of the streptococcus is located in the cell wall, whereas in group D it is found in the cytoplasm-protoplast membrane fraction (Jones and Shattock, 1960). It was thus of interest to examine the cell walls of the other groups to ascertain the location of the antigen. In all groups examined (Table 3), significant titers were observed. In several groups, however, weak agglutination was found. Similar results were obtained when the walls were tested against other sera or when HCl extracts were used as antigen in the precipitin test. Close correlation of the agglutination and precipitin tests was observed in all groups.

DISCUSSION

The presence of rhamnose in the cell walls of groups A–G (Cummins and Harris, 1956) has led to the recognition by some workers of rhamnose as a signature sugar among these microorganisms. The present results show that rhamnose is absent in one group and variable in three others. An examination of additional strains may reveal an even greater heterogeneity. The presence of rhamnose and the absence of other sugars in the great majority of strains in groups A and C serve to identify these two groups. In the other groups, however, the varied distribution of the carbohydrates does not aid in the identification of the groups.

The unclassifiable streptococci, owing in part to the presence of both arabinose and mannose, are more heterogeneous than most of the other groups. These strains, while at present serologically unclassified, may in the future yield other groups based on carbohydrate-containing determinants in the cell wall.

The cell walls of several genera of gram-positive bacteria have been shown to possess a mucopeptide structure composed of *N*-acetyl-muramic acid and alanine, glutamic acid, lysine, and glycine (Strominger, 1960; Perkins and Rogers, 1959). The presence of these compounds in strains from 11 serological groups of the streptococci (Table 2) and in groups A (Hayashi and Barkulis, 1959; Krause and McCarty, 1961) and C (Krause and McCarty, 1962) indicates that a similar mucopeptide unit is likely to be present in the cell walls of all streptococci. However, in contrast to groups A and C, in which rhamnose is the only sugar present, Table 1 shows that glucose, galactose, arabinose, and mannose are present, in addition to rhamnose, in the other groups. Consequently, the presence of these carbohydrates adds complexity and interest to the structure of the cell wall.

TABLE 3. *Agglutination of streptococcal cell walls with streptococcal-grouping antisera*[a]

Cell walls		Dilution of antisera (reciprocal)						
Group	Strain	20	40	80	160	320	640	Control
A	T1	2+	2+	2+	2+	2+	2+	−
B	Shipley	2+	2+	2+	2+	2+	−	−
	090R	2+	2+	2+	+	−	−	−
	Brownette	2+	2+	2+	2+	2+	+	−
	Lewis	2+	2+	2+	2+	+	+	−
C	26RP66	2+	2+	2+	+	−	−	−
D	D10	−	−	−	−	−	−	−
E	5385	2+	2+	2+	2+	2+	−	−
	K131	2+	2+	2+	2+	+	−	−
F	C628	2+	2+	2+	+	±	−	−
	DS1340	2+	2+	2+	−	−	−	−
	H60R	+	−	−	−	−	−	−
G	D166B	2+	2+	2+	2+	+	−	−
	Valente	2+	2+	2+	2+	+	−	−
	F68A	2+	2+	2+	+	−	−	−
H	Hockley	2+	2+	2+	2+	+	−	−
	Challis	2+	2+	2+	+	−	−	−
	SBE	2+	2+	2+	+	−	−	−
	F90A	2+	2+	2+	2+	2+	−	−
K	B1	2+	2+	2+	2+	+	−	−
	D34B	2+	2+	2+	2+	2+	2+	−
	D34E	2+	+	+	−	−	−	−
	GD	2+	+	+	−	−	−	−
	Murphy	+	±	−	−	−	−	−
L	D167A	2+	2+	2+	2+	2+	−	−
	D167B	2+	2+	2+	2+	2+	+	−
M	54X136	2+	2+	−	−	−	−	−
	54X134	+	−	−	−	−	−	−
N	B207	2+	2+	−	−	−	−	−
	B210	2+	2+	−	−	−	−	−
	C559	2+	2+	2+	2+	+	−	−
O	B361	2+	2+	2+	2+	−	−	−
	1360	2+	2+	2+	2+	2+	−	−
Q	R6566	2+	2+	−	−	−	−	−
	E6844	2+	2+	2+	+	−	−	−

[a] Antisera to groups A, B, D, E, F, K, L, M, and O were obtained from the U.S. Public Health Service, and group C from Lederle Laboratories. The remainder were prepared by the authors with the following strains; G-D166B, H-Hockley, K-D34E, N-C559, and Q-E6844. Symbols: 2+ = complete agglutination; + = partial agglutination; ± = trace.

The group-specific antigen in groups A–G is known to be carbohydrate in nature; no information is available on the remaining groups. It appears likely that the sugars found in the cell walls of the streptococci (Table 1) are a part of the group-specific antigen, which, in all cases except D, is in the cell wall (Table 3). The stereochemical arrangement of rhamnose, glucose, and galactose, and arabinose and mannose in a few cases, is probably responsible in part for the antigenic specificity of each of the groups. In addition, glucosamine and galactosamine (Table 2) are likely to be a part of the group carbohydrate antigen. Both of these amino sugars were present in each of the seven strains examined.

Fuller (1938) and Zittle and Harris (1942) demonstrated that the group carbohydrate antigen from strains of a number of serological groups of streptococci was solubilized in formamide at 160 C. The material was shown to be free of protein. Recent evidence obtained by this procedure (Slade and Slamp, 1962) shows that material containing the group antigen in two group K strains is composed of glucose, galactose, glucosamine, and galactosamine. Rhamnose may or may not be present. Similar findings were obtained with group O and an unclassified strain. In group A, the antigen is composed of rhamnose and glucosamine, and in group C, rhamnose and galactosamine (Krause and McCarty, 1961, 1962).

The structure of the grouping antigens in these organisms is of interest. The sugars and amino sugars probably form a structure, the long axis of which parallels the mucopeptide of the cell wall. The latter is considered to be the cytoskeleton of the wall and is responsible for the rigidity of the bacterial cell. The muramic acid of the mucopeptide may serve to link the group carbohydrate antigen, situated on or near the surface of the cell, with the cytoskeleton of the cell wall.

The streptococcal antigens, both group and type, may be composed of a polymer made up of a single sugar or several sugars with side chains composed of sugars and amino sugars. An amino sugar may be the terminal unit on the side chain. Variables such as the length and composition of the side chain, type of linkage between components, optical rotation of the sugars, and presence of α or β forms very likely are important to the specificity of these antigens. Many combinations must exist to explain the presence of 17 group antigens in the cell walls of streptococci, as well as 24 cell-wall-type antigens in group D

(Sharpe and Shattock, 1952), 4 in group E (Moreira-Jacob, 1956), 5 in group F (Ottens and Winkler, 1962), 2 in group H (Farmer, 1954), and many others yet to be detected.

The importance of the side chain and other aspects of chemical structure in the determination of the specificity of antigens in both gram-positive and gram-negative bacteria, and in mammalian cells, is apparent from the work of Goebel, Westphal, Staub, McCarty, Morgan, Kabat, and others (Ciba Foundation Symposium, 1958; Joshua Macy Jr. Foundation, 1959). In many cases, the sugars found in these antigens are the same as those in the cell walls of the streptococci, although other sugars are also present. The existence of compounds, other than those reported here, which could be a part of the group antigens of the streptococci must always be kept in mind.

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