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Studies of Streptococcal Cell Walls

VII. Carbohydrate Composition of Group B Cell Walls[1]

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ABSTRACT

WITTNER, MASAKO K. (Presbyterian-St. Luke's Hospital, Chicago, Ill.), AND JAMES A. HAYASHI. Studies of streptococcal cell walls. VII. Carbohydrate composition of group B cell walls. J. Bacteriol. **89**:398–402. 1965.—Group B streptococcal cell walls contain 63% protein, 10% rhamnose, 18% hexose (mainly galactose, but also some glucose), 7% hexosamine (mainly glucosamine, but also galactosamine), and 3% muramic acid. The group and type antigens were extracted from isolated cell walls by acid treatment and enzymatic hydrolysis, and fractionated either with ethanol or on a diethylaminoethyl-cellulose column. Serological and chemical analyses of the fractions obtained in the two fractionation methods show that the group antigen is a rhamnose-rich polysaccharide and that the type antigen is rich in galactose and contains hexosamines.

Group B streptococci (Lancefield, 1933), which are primarily bovine in origin, may be subdivided into four types by serological means (Lancefield, 1934, 1938). The group and type antigens have been shown to be polysaccharide in nature (Lancefield, 1938).

Investigations of the chemical composition of cell walls (Cummins and Harris, 1956; Slade and Slamp, 1962) have shown that the group B cell-wall carbohydrate constituents include rhamnose, glucose, and galactose, and the hexosamines glucosamine, galactosamine, and muramic acid. The amino acids alanine, lysine, and glutamic acid, which are characteristic components of the cell-wall mucopeptides of streptococci (Cummins and Harris, 1956), are also present in group B cell walls in large amounts.

This paper describes further studies on the chemical composition of group B cell walls. The group and type polysaccharides have been extracted from these cell walls and separated by chemical fractionation. The group activity has been found in association with a rhamnose-rich polysaccharide, and the type activity, with a polysaccharide rich in hexose, predominantly galactose.

MATERIALS AND METHODS

Organisms used and preparation of cell walls. The group B streptococcal strains used were obtained from the American Type Culture Collection and included the following strains: 12400 (type Ia, strain 090), 12401 (type Ib, strain H36B), 12973 (type II, strain V8), and 12403 (type III, strain D136C[3]). All strains were grown on Todd-Hewitt medium, following the method of Barkulis and Jones (1957), and cell walls were prepared by the method of Shockman, Kolb, and Toennies (1957), as modified by Hayashi and Walsh (1961). Cell walls were treated with crystalline trypsin, as described previously (Hayashi and Barkulis, 1959).

Analytical methods. Nitrogen was determined by a modified Nessler's reagent after digestion and distillation (Umbreit, Burris, and Stauffer, 1959). Methylpentose was measured by the cysteine-sulfuric acid method (Dische and Shettles, 1948), and hexosamine, by the method of Dische and Borenfreund (1950). Muramic acid was assayed by the same hexosamine method after its separation from glucosamine and galactosamine by the ion-exchange method of Strange and Dark (1956), by use of the factor of 0.55 for the relative color value of muramic acid compared with glucosamine. D-Glucose was assayed with a commercial glucose oxidase preparation (Worthington Biochemical Corp., Freehold, N.J.).

Total sugars were measured by the indole method of Dische, as described by Ashwell (1957). The relative color values of several sugars were measured, and, on a weight basis, the following values were found: L-rhamnose hydrate, 1.00; D-galactose, 0.70; D-glucosamine hydrochloride, 0.06; and D-glucose, 0.75.

Paper chromatograms were run in a one-dimensional, ascending system with use of butanol-acetic acid-water (12:3:5) or ethyl acetate-acetic

[1] Taken in part from a thesis submitted in partial fulfillment of the requirements for the M.S. degree in the Graduate College, University of Illinois College of Medicine.

acid-water (3:1:3), and the spots were made visible with aniline hydrogen phthalate or Elson-Morgan sprays (Smith, 1960). Recrystallized samples of commercially available sugars were used as reference compounds. A muramic acid reference sample was obtained from group A streptococcal cell walls by acid hydrolysis and fractionation on a Dowex-50 column (Hayashi and Barkulis, 1959) and on a Dowex-50-phosphate column at *p*H 6.5 (Strange and Dark, 1956).

Serological methods. Group and type antisera were obtained by immunizing rabbits intramuscularly with whole formalinized cells in saline or cell walls in incomplete Freund adjuvant, following procedures described previously (Hayashi and Walsh, 1961). Assays for serological activity were made by use of tube precipitin tests (Lancefield, 1947).

RESULTS

Chemical analyses. The results of analyses of group B cell walls are shown in Table 1. These results indicate that group B streptococcal cell walls contain 10% rhamnose, 18% hexoses (mainly galactose, but also a small amount of glucose), and about 9% hexosamine, which is mainly glucosamine, although some galactosamine was detected on paper chromatograms. Group B streptococcal cell walls are, therefore, similar in chemical composition to other streptococcal cell walls.

Separation of antigens by ethanol fractionation. Lancefield (1934) separated the group and type antigens by fractionation with ethanol, and showed their carbohydrate character. The same method was used in this study, with some slight modifications, to effect a separation of the group and type antigens.

Group B type II cell walls (449 mg) were suspended in 45 ml of 0.9% saline and the *p*H was adjusted to 2.0 with dilute HCl solution. The suspension was heated in a boiling-water bath, with stirring, for 10 min. After clarification of the suspension by centrifugation, the residue was re-extracted, by use of 45 ml of the acidified saline. The two extracts were combined and adjusted to *p*H 7.0 with dilute NaOH solution. A precipitate which formed at this point was discarded, and 3 volumes of 95% ethanol were added to the supernatant solution. The resulting precipitate was separated by centrifugation, redissolved in 50 ml of saline, and reprecipitated with 3 volumes of ethanol. The supernatant solution from the first ethanol precipitation was concentrated in vacuo to remove ethanol, and then treated again with 3 volumes of ethanol. The supernatant solutions from the latter two precipitations were combined, dialyzed against water, and lyophilized, yielding 51 mg of solid material, which was unreactive serologically. The combined precipitates weighed 56.8 mg and showed both group and type activity.

The serologically active material was dissolved in 50 ml of saline, and 100 ml of 95% ethanol were added with stirring. The precipitated material (21.2 mg) showed weak group and type

TABLE 1. *Carbohydrate composition of group B streptococcal cell walls*[a]

Streptococcal strains	Wt loss after trypsin treatment	N	Total aldose[b]	Rhamnose hydrate	Total amino sugar[c]	Muramic acid[d]	Total hexosamine[e]	Total hexose[f]	Glucose[g]
	%	%							
Type Ia									
Intact	39.9	10.0	28.0	10.0	9.8	2.9	8.4	18.0	2.4
Trypsin-treated	—	7.3	48.2	15.9	13.0	5.0	10.6	32.3	3.0
Type Ib									
Intact	38.9	10.7	27.0	10.2	10.8	3.1	9.1	16.8	1.1
Trypsin-treated	—	8.0	45.5	16.9	15.2	5.0	12.8	28.6	1.8
Type II									
Intact	40.4	10.2	26.9	10.0	11.0	2.9	9.6	16.9	1.9
Trypsin-treated	—	7.4	45.8	16.4	15.4	4.8	12.9	29.4	2.9
Type III									
Intact	40.1	10.5	28.6	11.0	9.9	3.0	8.4	18.6	1.2
Trypsin-treated	—	7.7	47.9	15.5	12.8	4.9	10.5	32.4	1.0

[a] Values given as milligrams of component liberated per 100 mg of cell walls except where indicated.
[b] Analysis by indole method with L-rhamnose as standard.
[c] Values as glucosamine hydrochloride.
[d] By the method of Strange and Dark (1956) and the ratio (color value of 1 μM muramic acid)/(color value of 1 μM glucosamine HCl) = 0.55.
[e] Calculated by: (total amino sugar) − (muramic acid color value) = total hexosamine.
[f] Calculated by: (column 3) − (column 4) = (column 8).
[g] Analyzed by use of glucose oxidase.

TABLE 2. *Carbohydrate composition and serological activity of polysaccharide fractions from group B type II cell walls*

Polysaccharide fraction	Dry wt	Sugars present[a]	Per cent carbohydrate[b]	Per cent rhamnose	Serological activity	
					Group B	Type II
	mg					
Ethanol precipitation						
Starting material[c]	56.8	—	35.5	30	4+	3+
71% ethanol-soluble	16.2	—			4+	3+
71% ethanol-insoluble	11.2	Galactose Glucosamine Galactosamine	72	0	0	2+
DEAE-cellulose						
Starting material[d]	40.0	—	10		4+	4+
Peak I	19.8	Rhamnose glucosamine	22	14.3	4+	0
Peak II	11.0	—	26	1.3	2+	1+
Peak III	2.5	—	31	1.1	0	2+
Peak IV	4.3	—	44	1.1	0	1+

[a] By paper chromatography.
[b] By indole method (Ashwell, 1957).
[c] Prepared by heating at *p*H 2 (Lancefield, 1934).
[d] Prepared with use of *Streptomyces albus* enzyme complex (McCarty, 1952).

activity, whereas the material recovered from the supernatant solution (17.9 mg) showed strong group and type activity.

Both fractions were combined and dissolved in 10 ml of saline, and the solution was treated with 2.5 volumes of ethanol. The resulting amounts of solid material were 11.0 mg from the supernatant solution and 16.2 mg from the precipitate. The supernatant material showed strong group B activity with some type II activity; the precipitate showed only type activity. Chemical analyses of the type-reactive material showed a carbohydrate content of 72%, with little or no rhamnose present. Paper chromatograms showed two spots corresponding to galactose and glucosamine.

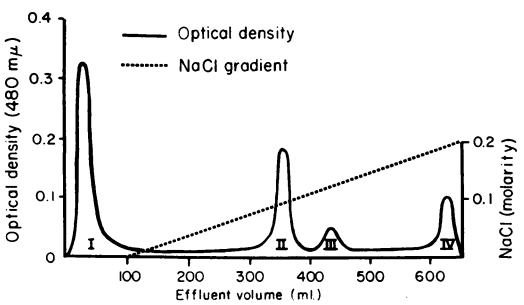

FIG. 1. *Chromatographic separation of extracted group and type antigens of group B cell walls. Fractions (5 ml) were collected and 0.1-ml portions of each fraction were analyzed for total hexose (Ashwell, 1957). Carbohydrate composition of each peak is given in Table 2.*

The results (Table 2) indicate that type activity is associated with a polysaccharide containing galactose and hexosamine, whereas the group-reactive material has a high rhamnose content.

Ethanol fractionation of cell-wall polysaccharides prepared by the formamide method (Fuller, 1938) gave the same separation of group and type activity.

Separation of group and type antigens on diethylaminoethyl (DEAE) cellulose. Further verification of the results obtained by ethanol fractionation was obtained by using a different method of fractionation. The polysaccharide fraction used as starting material was obtained from a partial hydrolysate of group B, type II cell walls, by use of *S. albus* enzyme, a commercial preparation obtained from Consolidated Laboratories, Inc., Chicago Heights, Ill. (McCarty, 1952). The polysaccharide fraction (40 mg; 10% carbohydrate) was dissolved in 5 ml of 0.05 M borate buffer (*p*H 8.5), and added to a column (1 by 20 cm) of DEAE cellulose (Brown Paper Co., Keene, N.H.), equilibrated with the same buffer. After the passage of 100 ml of the buffer through the column, elution was continued by imposing a linear gradient of NaCl up to 0.2 M over a total volume of 500 ml of the borate buffer. Fractions (5 ml) were collected, and 0.1 ml from each fraction was analyzed for total

carbohydrate. An elution diagram is shown in Fig. 1, and the results of chemical and serological analyses are given in Table 2. The results in the table show that group activity is associated with the rhamnose polysaccharide, and that type activity is primarily found in the galactose-rich fraction.

DISCUSSION

The results of the chemical analyses presented here confirm that group B cell walls are similar in composition to other streptococcal cell walls and cell walls of other gram-positive organisms (Salton, 1960).

Slade and Slamp (1961) showed by agglutination tests that the group antigen is found in the cell walls of streptococci. We have shown by direct extraction and isolation that the group B activity is directly associated with a rhamnose, polysaccharide of the cell wall, and, furthermore, that the type activity is associated with a separate and distinct polysaccharide which contains a high proportion of hexoses, mainly galactose. The data presented here do not show, however, whether these sugars, rhamnose and galactose, are the primary determinants of the serological specificity shown by the polysaccharides from the cell wall. McCarty (1956) has shown that group A serological activity is found in a rhamnose polymer containing *N*-acetyl-D-glucosamine side-chains, and Krause and McCarty (1962) have shown that group C activity is dependent upon galactosamine side-chains on the rhamnose-containing group polysaccharide. A report published recently (Curtis and Krause, 1964) indicates that the group B serological reaction appears to be inhibited specifically by solutions of the monosaccharide L-rhamnose. The present data, showing that the group antigen is rich in rhamnose, are consistent with the serological data, indicating that rhamnose is the serologically determinant sugar in the group B antigen.

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LITERATURE CITED

ASHWELL, G. 1957. Colorimetric analysis of sugars, p. 73. *In* S. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 3. Academic Press, Inc., New York.

BARKULIS, S. S., AND M. F. JONES. 1957. Studies of streptococcal cell walls. I. Isolation, chemical composition, and preparation of M protein. J. Bacteriol. **74**:207–216.

CUMMINS, C. S., AND H. HARRIS. 1956. The chemical composition of the cell wall in some gram-positive bacteria and its possible value as a taxonomic character, J. Gen. Microbiol. **14**:585–600.

CURTIS, S. M., AND R. M. KRAUSE. 1964. Identification of rhamnose as an antigenic determinant of group B streptococcal carbohydrates. Federation Proc. **23**:191.

DISCHE, Z., AND E. BORENFREUND. 1950. A spectrophotometric method for the microdetermination of hexosamines. J. Biol. Chem. **184**:517–522.

DISCHE, Z., AND L. B. SHETTLES. 1948. A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem. **175**:595–603.

FULLER, A. T., 1938. Formamide method for the extraction of polysaccharides from hemolytic streptococci. Brit. J. Exp. Pathol. **19**:130–139.

HAYASHI, J. A., AND S. S. BARKULIS. 1959. Studies of streptococcal cell walls. III. The amino acids of the trypsin-treated cell wall. J. Bacteriol. **77**:177–184.

HAYASHI. J. A., AND G. WALSH. 1961. Studies of streptococcal cell walls. VI. Effects of adjuvants on the production of type-specific antibodies to cell walls and isolated M protein. J. Bacteriol. **82**:736–742.

KRAUSE, R. M., AND M. MCCARTY. 1962. Studies on the chemical structure of the streptococcal cell wall. II. The composition of group C cell walls and chemical basis for serologic specificity of the carbohydrate moiety. J. Exp. Med. **115**: 49–62.

LANCEFIELD, R. C. 1933. A serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med. **57**:571–595.

LANCEFIELD, R. C. 1934. A serological differentiation of specific types of bovine hemolytic streptococci (group B). J. Exp. Med. **59**:441–458.

LANCEFIELD, R. C. 1938. Two serological types of group B hemolytic streptococci with related, but not identical, type-specific substances. J. Exp. Med. **67**:25–40.

LANCEFIELD, R. C. 1947. Technical instructions and procedures employed in the production of streptococcus grouping and typing sera. Brochure of the Rockefeller Institute for Medical Research, New York.

MCCARTY, M. 1952. The lysis of group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. I. Production and fractionation of the lytic enzymes. J. Exp. Med. **96**:555–568.

MCCARTY, M. 1956. Variation in the group-specific carbohydrate of group A streptococci. II. Studies on the chemical basis for serological specificity of the carbohydrate. J. Exp. Med. **104**:629–643.

SALTON, M. R. J. Microbial cell walls. John Wiley & Sons, Inc., New York, 1960.

SHOCKMAN, G. D., J. J. KOLB, AND G. TOENNIES. 1957. A high-speed shaker for the disruption of cells at low temperature. Biochim. Biophys. Acta **24**:203–205.

SLADE, H. D., AND W. C. SLAMP. 1962. Cell-wall composition and the grouping antigens of streptococci. J. Bacteriol. **84**:345–351.

SMITH, I. 1960. Chromatographic and electrophoretic techniques, vol. 1. Interscience Publishers, New York.

STRANGE, R. E., AND F. A. DARK. 1956. An unidentified amino sugar present in cell walls and spores of various bacteria. Nature **177**:186–188.

UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1959. Manometric techniques. Burgess Publishing Co., Minneapolis.