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Chemical and Serological Relationships Between the Heteropolysaccharides of *Mycobacterium tuberculosis* and *Mycobacterium kansasii*[1]

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The identity of a heteropolysaccharide from cell walls of *Mycobacterium tuberculosis* H37Ra with Seibert's tuberculopolysaccharide I was demonstrated by thin-layer chromatography, chemical analysis, and antigenic tests. The polysaccharide of *M. kansasii* was shown to be identical with that of *M. tuberculosis*. Defatted cells were disintegrated by ultrasonic treatment in the presence of glass beads; cell walls were obtained by differential ultracentrifugation. Ethyl alcohol-precipitated carbohydrate extracts were analyzed for protein and nucleic acid; these impurities were removed. Tuberculopolysaccharide I from the mycobacterial culture filtrate is probably derived from a lipopolysaccharide of the cell wall, which is partially removed by chloroform in the intact state. Alkaline extraction releases additional polysaccharide, in varying degrees of association with cell wall murein.

The complex chemical composition and metabolic products of mycobacteria have been studied for many years in order to better comprehend the immune response in tuberculous disease. Cross-reactions between *Mycobacterium tuberculosis* and the closely related atypical mycobacteria have been noted in skin testing and in serological studies. The purpose of the present study was to examine the chemical and antigenic relationships between *M. tuberculosis* H37Ra and *M. kansasii*.

The literature contains descriptions of at least eight polysaccharides, which have been classified into three categories: somatic polysaccharides, lipopolysaccharides extracted from the unbroken cell, and tuberculopolysaccharides of the culture filtrate.

Tuberculopolysaccharide I is one of two antigenic polysaccharides isolated from the culture filtrate of mycobacteria by Seibert (10). It is a heteropolysaccharide containing arabinose, galactose, and mannose, with a molecular weight of 7,000 to 9,000. Connected to a nitrogenous residue, tuberculopolysaccharide I closely resembles the lipid-bound somatic polysaccharide characterized by Haworth and Stacey (5) and the murein-associated cell wall polysaccharide described by Cummins (3).

The origin of the culture filtrate tuberculopolysaccharides is not known. One theory is that they are derived from cell wall macromolecules, rather than being exogenous by-products (12). Results obtained in this study support this idea as well as the concept that the polysaccharide is present as a common antigen in most mycobacterial cells (2, 6, 8).

MATERIALS AND METHODS

Source of organisms. The H37Ra and *M. kansasii* strains were obtained from the Tuberculosis Laboratory, Communicable Disease Center, Atlanta, Ga., and have been passed continuously in our laboratory. Cells were grown in Proskauer-Beck medium at 37 C for 6 to 8 weeks before harvesting.

Preparation of antigens. Tuberculopolysaccharide I was isolated from filtered Proskauer-Beck growth medium by Seibert's method (10). Chromatography on Sephadex G200 indicated that the compound was homogenous.

The procedure for isolation of the somatic polysaccharide is summarized in Fig. 1. To obtain cell walls, bacteria were first defatted by an adaptation of Anderson's method (1). Twenty volumes of ethyl alcohol-ether (1:1) were mixed with the cells for 24 hr. Twenty volumes of chloroform were agitated with the residue for 24 to 48 hr; 20 volumes of ethyl alcohol-ether

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MYCOBACTERIAL CELLS

↓ Ethyl alcohol:ether 20 volumes 24 hr
Chloroform 20 volumes 24 hr
Ethyl alcohol:ether:HCl 20 volumes 24 hr

DEFATTED MYCOBACTERIAL CELLS

↓ Sonic treatment with glass beads
Differential ultracentrifugation
Trypsin

CELL WALLS + CYTOPLASM

↓ Alkali extraction 90 C
Purification—Precipitation with alcohol dialysis; repeat PPT + dialysis protein removal

SOMATIC POLYSACCHARIDE EXTRACT

FIG. 1. *Isolation of mycobacterial polysaccharides.*

(1:1) with 1% HCl were stirred with the remaining cells for 24 hr.

From the chloroform extract, a lipopolysaccharide was isolated by the method of Anderson (1). Its polysaccharide moiety, used in Ouchterlony tests (Fig. 3), was obtained by mild alkaline hydrolysis.

The defatted cells (600 mg, wet weight, per 20 ml of water) were then disintegrated by ultrasonic treatment with a Raytheon Magnetostriction oscillator and Ballotini beads, for 1.5 hr. The crude extract was subjected to differential centrifugation according to Salton's scheme (9).

The somatic polysaccharide was prepared by a modification of Haworth's method (4). Cell walls were refluxed at 85 C for 24 hr with two volumes of 1.0 N NaOH, followed by acidification to *p*H 4.5. After dialysis of the supernatant fraction, it was concentrated by rotary evaporation at 80 C. Fractions were obtained by precipitation with 95% ethyl alcohol; the later fractions contained a higher percentage of the heteropolysaccharide with lower concentrations of glucose, as well as protein and nucleic acids. The fractions used were purified by repeated dialysis and reprecipitation. They were concentrated by rotary evaporation at 80 C. Protein and nucleic acid impurities were removed by Sevag's procedure (13) or by use of cetyl ammonium bromide (17). Carbohydrate and protein concentrations of the fractions used are summarized in Table 1.

Methods of chemical analysis. The procedures used are all described in the methodology manual published by the National Tuberculosis Association (11). Protein was analyzed by a modification of the Lowry-Miller method. Nucleic acids were examined by adaptation of the Dische (diphenylamine) and Bial (orcinol) tests. Carbohydrate concentration was measured by the anthrone test. The presence of protein was also studied by disc electrophoresis and by ultraviolet spectrophotometry.

TABLE 1. *Carbohydrate and protein content of polysaccharide fractions of Mycobacterium tuberculosis and M. kansasii*

Organism	Fraction	Carbohydrate calculated as glucose	Total solids calculated as protein
		mg/ml	%
M. kansasii	Somatic polysaccharide	4.5	1.6
	Tuberculopolysaccharide I	17.6	0.9
M. tuberculosis	Somatic polysaccharide	16.9	0.8
	Tuberculopolysaccharide I	1.0	2.5

Thin-layer chromatography. To prepare the samples for thin-layer chromatography, samples were evaporated to contain about 50 mg of sugar per ml of water, and 1 ml of HCl (1.0 N) or H_2SO_4 (2.0 N) was added to each. These fractions were placed in small lyophilizing ampoules which were flame-sealed at the open end. After boiling in water for 6 hr, the fractions were neutralized and salts were removed.

The following ascending systems gave good identification of sugars. (i) Phenol-water (90:10) on cellulose by the method of Vomhof and Tucker (15). Plates were chromatographed twice for 3 hr in a sandwich chamber. (ii) Propanol-ammonia-water (60:20:10) on a 250-mμ silica gel layer prepared with 0.1 N boric acid (25 g of silica per 50 ml of solution) and run in two dimensions in a saturated chromatography trough (2 hr each direction). (iii) A two-dimensional saturated trough using butanol-acetone-water (40:50:10) in one direction, and propanol-ethyl acetate-water (70:20:10) in the other, on Eastman silica chromatograms (1 hr each direction).

Aniline phthalate or anisaldehyde sprays were used to reveal sugars as summarized by Waldi (16). Hexosamines were separated on silica impregnated with borate, using a solvent of pyridine-ethyl acetate-acetic acid-water (5:5:1:3). Spots were demonstrated by spraying with the Elson-Morgan reagent (16).

Antisera. Adult white male New Zealand rabbits were injected weekly with an antigenic mixture of equal amounts of disintegrated cells (30 mg/ml) and culture filtrate diluted with Freund's adjuvant (6 ml of antigen/20 ml of adjuvant). Four out of five rabbits gave good antisera with the following immunization schedule: day 1, 1 ml of antigenic mixture in Freund's, subcutaneously; day 7, 0.5 ml of antigenic mixture in Freund's, subcutaneously; day 14, 0.1 ml of antigenic mixture, intraperitoneally (4 mg/ml) without Freund's; day 28, test bleed. Rabbits which did not have high antibody titers were rested for 2 weeks and

then injected with 0.2 ml of antigenic mixture subcutaneously; after 1 week, 0.1 ml was injected subcutaneously, if necessary.

Rabbits were bled by venipuncture 1 week after the last injection. After clotting, the clear serum was removed, centrifuged, and frozen in small quantities for later use.

Qualitative gel precipitation. A modification of the Oakley-Fulthorpe method (7) was used. By micropipette, antiserum was added to Parlett tubes to the 2-cm mark. A warm, filtered, 0.3% Noble agar solution (in 0.85% saline with 10^{-4} M Merthiolate) was carefully layered over the serum to the 3-cm mark. When this had solidified, antigen was added to the 5-cm mark. Five dilutions of each antigen were used, with control serum and saline. The tubes were sealed, placed upright in cardboard racks within a closed chamber, and examined daily for 1 week. The quantity, width, and sharpness of precipitating bands were optimum after 3 to 5 days.

Ouchterlony double gel diffusion. About 5 ml of a filtered solution of 0.75% Noble agar was added to 0.85% NaCl and 0.5% sodium azide in distilled water on dichromate-cleaned lantern slides. After solidification of the agar, templates were used for duplicate patterns of six circular wells around a central well. The wells were evenly filled with antigen or antiserum by micropipette at the start of the diffusion only, and the slides were placed in a covered humid chamber at room temperature. Good results were obtained within 18 to 36 hr.

RESULTS

The present study indicates that tuberculopolysaccharide I, prepared from the culture filtrate of either the H37Ra strain or of *M. kansasii*, is derived from the homologous somatic polysaccharide. The conclusion that the two polysaccharides are identical is based upon three types of evidence: examination of the quantitative sugar distribution in the compounds as shown by their spectrophotometric ratios; comparison of their qualitative sugar composition by thin-layer chromatography; and serological studies.

The ratio of values given by the anthrone test at wavelengths of 600 λ roughly reflects the distribution of the characteristic sugars of a polysaccharide (11). The average anthrone ratio for pure tuberculopolysaccharide I from *M. tuberculosis*, with its arabinose, galactose, and mannose, is 1.6. In Table 2, data are presented to show that the anthrone ratio of the somatic polysaccharide is almost the same as that of tuberculopolysaccharide I.

In all of the compounds studied, arabinose and galactose were identified as main components. Mannose was seen in some preparations. In Fig. 2, a typical chromatogram from the somatic polysaccharide of *M. tuberculosis* is seen, showing the three sugars. The presence of acetylglucosamine as one of the two sugar amines, demonstrated by their differential color reaction with aniline phthalate, was confirmed by the Elson-Morgan reaction.

TABLE 2. *Comparison of anthrone ratios, 600λ/540λ, tuberculopolysaccharide I and somatic polysaccharide*

Organism	Lot no.	Somatic polysaccharide	Tuberculopolysaccharide I
Mycobacterium tuberculosis	135	1.6	—
	158	1.6	1.7
	161	1.4	—
M. kansasii	1	—	1.8
	2	1.7	—
	3	1.9	1.8

Antigenic studies were done to ascertain whether tuberculopolysaccharide I is indeed identical to the homologous somatic polysaccharide in a given organism, and also to establish the serological relationship of the polysaccharides of *M. tuberculosis* and *M. kansasii*.

Preliminary studies, to show antigenicity of the compounds by using the Oakley-Fulthorpe technique of double diffusion in tubes, are diagrammed in Table 3. Precipitin reactions are shown with increasing antigenic dilutions expressed as milligrams of glucose. With either the somatic polysaccharide or tuberculopolysaccharide I as antigen against homologous antiserum, a single typical band was seen near the base of the agar gel. This band was the lowest of five bands shown with the sensitizing antigenic mixture. The polysaccharide band was not seen with normal rabbit sera, but was seen with heterologous sera.

Antigenic studies, using Ouchterlony plates, are typified in Fig. 3 and 4. The development of a band of identity between tuberculopolysaccharide I and the somatic polysaccharide of H37Ra, along with polysaccharide from the lipopolysaccharide extracts, against homologous antiserum is pictured in Fig. 3. A similar band of identity was also seen between the polysaccharide I and the somatic polysaccharide derived from *M. kansasii* against its own antiserum.

In Fig. 4, the somatic polysaccharides of *M. kansasii* and *M. tuberculosis*, represented in wells A and B, share a band of identity, which indicates that the somatic polysaccharide is common to the two organisms. This was shown by using each antiserum against either strain in the center well against homologous and heterologous antigens. Periodic acid-Schiff's carbohydrate stain confirmed that polysaccharide was in the bands.

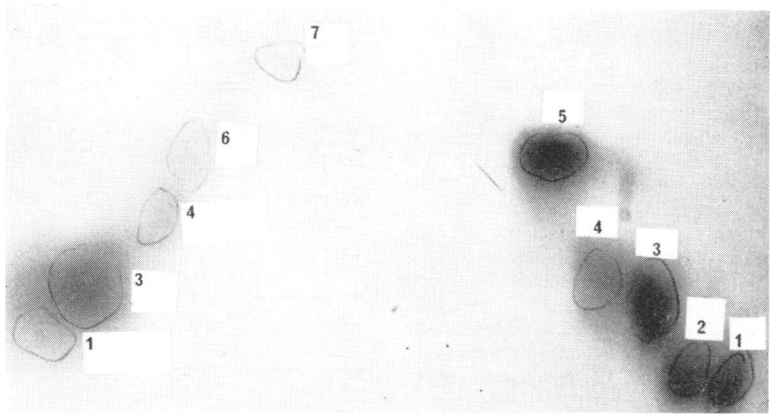

FIG. 2. *Chromatogram of sugars from Mycobacterium tuberculosis H37Ra somatic polysaccharide: (1) galactose; (2) glucose; (3) arabinose; (4) mannose; (5) rhamnose; (6) acetylglucosamine; (7) hexosamine (not yet identified).*

TABLE 3. *Precipitin reactions of polysaccharides; Oakley-Fulthorpe tube test*

Dilutions as milligrams of carbohydrate	20-10 mg	10-5 mg	5-1 mg	1 mg-500 γ	500-100γ	100-50γ	50-10γ	10-1γ
Homologous serum								
Mycobacterium kansasii								
Somatic polysaccharide			−	+	++	++	+	+
Polysaccharide I	−	−	−	+	+	++	+	
M. tuberculosis								
Somatic polysaccharide	−	−	+	+	+	+	++	
Polysaccharide I			+	+	++	+	+	+
Heterologous serum								
M. kansasii								
Somatic polysaccharide (with *M. tuberculosis* antiserum)			−	+	+	++		
M. tuberculosis								
Polysaccharide I (with *M. kansasii* antiserum)					++			

Control tests by immune adsorption were done with both Ouchterlony and Oakley-Fulthorpe techniques. Addition of the optimal proportion of the *M. kansasii* somatic polysaccharide to H37Ra antiserum prevented the reactions with H37Ra somatic polysaccharide, lipopolysaccharide, and tuberculopolysaccharide I. This indicates that the specific precipitins for the common antigen had been removed.

DISCUSSION

The presence of the somatic heteropolysaccharide as a common group antigen in *M. kansasii* and *M. tuberculosis* agrees with the closely related chemical composition of many mycobacterial cell walls established by Cummins (3). The culture filtrate tuberculopolysaccharide I seems to be either a precursor of the somatic heteropolysaccharide or a breakdown product from the somatic cell wall.

In addition to the chemical and immunological evidence for the identity of tuberculopolysaccharide I and the somatic heteropolysaccharide, it must be noted that the somatic polysaccharide is precipitated in late alcohol fractions during its isolation. In Seibert's alcohol fractionation procedure, tuberculopolysaccharide I is also the last fraction to precipitate from the culture filtrate.

Both tuberculopolysaccharide I and the somatic polysaccharide have been found to be connected to a nitrogenous residue of variable length, which has been identified as glucosamine alone, or as those amines and amino acids comprising the mucopeptide complex (3, 14, 18, 19). As with any long-chain macromolecule, experimental degradation or variations in growth media and enzymatic

pathways can result in differences in physical properties and chemical composition.

Some workers (2, 8) have found that tuberculopolysaccharide I is similar chemically and antigenically to the lipopolysaccharide, Wax D, first described by Anderson (1). Wax D has also been found connected to murein components (14). Definitive studies are still needed to compare antigenicity and quantitative sugar composition in these two compounds. The somatic polysaccharide may occur in the cell wall linked to lipid as well as to the mucopeptide complex.

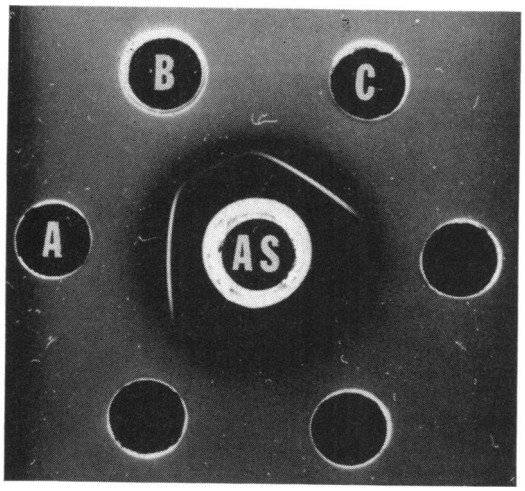

FIG. 3. *Bands of identity between H37Ra tuberculopolysaccharide I, the lipopolysaccharide, and the somatic polysaccharide of H37Ra against homologous antiserum. (A) Mycobacterium tuberculosis H37Ra somatic polysaccharide; (B) H37Ra lipopolysaccharide-polysaccharide moiety; (C) H37Ra tuberculopolysaccharide I; (AS) antiserum to M. tuberculosis H37Ra.*

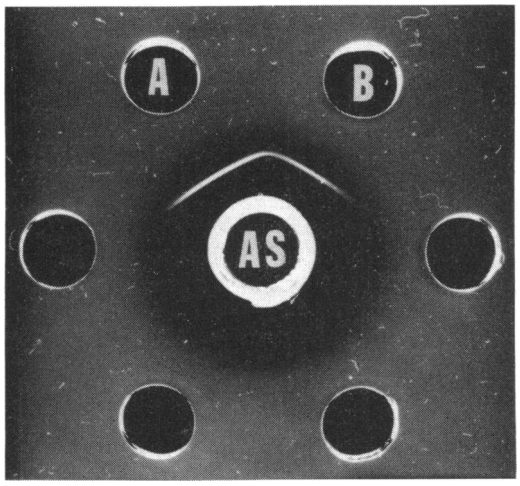

FIG. 4. *Band of identity between the somatic polysaccharide of Mycobacterium kansasii and that of M. tuberculosis H37Ra against H37Ra antiserum (18 hr). (A) Somatic polysaccharide of M. kansasii; (B) somatic polysaccharide of M. tuberculosis H37Ra; (AS) M. tuberculosis H37Ra antiserum.*

Studies are in progress to compare the chemical composition and antigenic relationship of the polysaccharides of representative atypical mycobacteria from Runyon's group II and group III. In addition, work is under way to characterize further the heteropolysaccharide observed in lipid extracts, and to investigate the biological effects of the heteropolysaccharides.

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