IMMUNOLOGICALLY ACTIVE POLYSACCHARIDES FROM *NOCARDIA ASTEROIDES* AND *NOCARDIA BRASILIENSIS*

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Received for publication 2 October 1962

ABSTRACT

ZAMORA, A. (University of Mexico, Mexico, D.F.), L. F. BOJALIL, AND FERNANDO BASTARRACHEA. Immunologically active polysaccharides from *Nocardia asteroides* and *Nocardia brasiliensis*. J. Bacteriol. **85**:549–555. 1963.—Two immunologically active polysaccharides were isolated from *Nocardia asteroides* (Poly I Na and Poly II Na) and *N. brasiliensis* (Poly I Nb and Poly II Nb). These polysaccharides were isolated from cell extracts and purified by methanol precipitation, chloroform extraction of extraneous material, and deproteinization with trichloroacetic acid. The crucial step used for separation of Poly I and Poly II from both nocardias was differential solubility. From dried preparations containing both polysaccharides, Poly I was solubilized at pH 10, whereas Poly II remained insoluble and was subsequently solubilized at pH 5. Poly I Na and Poly I Nb are apparently the same. Arabinose and galactose were the monosaccharide constituents of these polysaccharides, and their molar ratios were similar. Furthermore, Poly I Na and Poly I Nb cross-reacted in agar diffusion precipitin tests with rabbit antisera prepared against either *N. asteroides* or *N. brasiliensis*. Either polysaccharide absorbed serum antibodies against the other. These polysaccharides can be regarded as group-specific. Poly II Na and Poly II Nb are different and species-specific. They are composed of arabinose, galactose, and mannose but exhibit different molar ratios of these sugars according to species. They reacted only with homologous antisera.

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Previous investigations conducted in our laboratory were concerned with the differentiation between strains of *Nocardia asteroides* and *N. brasiliensis* by means of physiological tests (Bojalil and Cerbon, 1959; Bojalil, Trujillo, and Cerbon, 1959). To inquire into the antigenic relationships of these microorganisms, we felt it of interest to extend our experiments to the study of their polysaccharide constituents.

Studies on the polysaccharide components of species of *Nocardia* are rather scanty. González-Ochoa and Vázquez-Hoyos (1953) isolated polysaccharides of unknown composition from *N. asteroides* and *N. brasiliensis*. To our knowledge, Bishop and Blank (1958) have been the only ones to characterize chemically a somatic polysaccharide from *N. asteroides*. This paper deals with the purification and immunological characterization of somatic polysaccharides from *N. asteroides* and *N. brasiliensis*.

MATERIALS AND METHODS

Microorganisms and cultural conditions. Three strains of *N. asteroides*, ISET-23, ISET-1160, and UPHG-121, as well as three strains of *N. brasiliensis*, UPHG-23, UPHG-24, and UPHG-39, were used. The description of the morphological and physiological features of most of these strains as well as the methods used in their classification have been previously reported (Bojalil and Cerbon, 1959; Bojalil et al., 1959). Cells were grown for 21 days in a modified Proskauer and Beck medium (Youmans and Karlson, 1947) and defatted with a mixture of methanol-acetone (3:2) for 24 hr by use of 250 ml of the solvent for every 15 to 20 g (dry wt) of cells.

Analytical methods. Total nitrogen was determined by a micro-Kjeldahl procedure. Protein was determined by a biuret method described by Weichselbaum (1946) and modified by Ditterbrandt (1948). Phosphorus was determined according to Fiske and SubbaRow (1925). The

reducing sugar content of the polysaccharides was estimated by the colorimetric method of Somogyi (1952), using glucose as a standard; the polysaccharides were first hydrolyzed with 1 N H_2SO_4 for 1 to 7 hr at 100 C and then neutralized by Amberlite IR-4B (Rohm & Haas Co., Philadelphia, Pa.).

Individual sugars were identified by one-dimensional descending paper chromatography. Samples (20 mg) of the polysaccharides were hydrolyzed in sealed tubes for 6 hr at 100 C with 5 ml of 1 N H_2SO_4, and then neutralized by Amberlite IR-4B. Hydrolysates were applied to Whatman no. 1 paper. The solvent used was *n*-butanol-ethyl acetate-acetic acid-water (30:30:6:10; v/v). The chromatograms were developed for 72 hr, and the sugar spots detected with diphenylamine-aniline (Buchan and Savage, 1952; Harris and MacWilliam, 1954).

Quantitative determinations of monosaccharides were carried out after localization of the sugar spots by eluting parallel sugar areas with water at 45 C for 40 min. This was followed by a colorimetric determination (Somogyi, 1952), using samples of the corresponding sugars to prepare standard curves. Sharp separation of arabinose and mannose was accomplished by using the multiple-development technique of Jeanes, Wise, and Dimler (1951).

Preparation of antisera. Antisera were prepared against *N. asteroides* ISET-20 and *N. brasiliensis* UPHG-24. Three rabbits were inoculated per strain. All rabbits received, at each of four subcutaneous sites, 1 mg of defatted cells homogenized in 0.5 ml of a mixture of Bayol 55 (Esso Standard Oil Co., New York, N.Y.) and commercial lanolin (9:1). This procedure was repeated twice more at 1-week intervals. Each animal received, 2 weeks after the last subcutaneous inoculation, three intraperitoneal inoculations of 1 mg of defatted cells suspended in saline at 1-week intervals. The rabbits were bled from the heart 10 days after the last injection. The sera obtained were preserved at 0 C after the addition of Merthiolate to a final concentration of 1:10,000.

Antisera absorption. Immune sera were absorbed with polysaccharides from either homologous or heterologous *Nocardia*; 1 ml of the serum to be absorbed was added to 1 ml of 0.02 M phosphate buffer (pH 7.2) containing a slight excess of polysaccharide antigen. The mixture was stored for 1 week at 4 C. The supernatant was removed and tested by the agar gel diffusion method.

Precipitin reactions. Ouchterlony's (1949) agar gel diffusion technique was employed. After the agar had hardened, wells were cut out with a cork borer and sealed at the bottom with one drop of molten agar; 0.1 ml of antiserum was deposited in the center well, and 0.1 ml of the appropriate antigen was placed in the peripheral wells. The polysaccharide antigens used were adjusted to contain 50 mg of purified material in 100 ml of 0.02 M phosphate buffer (pH 7.2). Antiserum and antigens were placed in the wells without previous mixing with molten agar. The plates were incubated at 21 C for up to 72 hr in a moist chamber.

RESULTS

Preparation of polysaccharides. Defatted cells (20 g) were ground with 40 g of Pyrex glass powder for 90 min in a mortar, and the mixture was suspended in 250 ml of 3 M KCl. The suspension was centrifuged for 15 min at 800 × *g*. The supernatant was centrifuged twice for 2 hr (each time at 3,500 × *g*) to eliminate unbroken cells and debris. Ten volumes of methanol were added to the cell-free supernatant, and the mixture was left standing for 24 hr at 4 C. The mixture was then centrifuged, and the precipitate resuspended in 50 ml of 0.02 M phosphate buffer (pH 7.2). This suspension was dialyzed against running tap water for 72 hr and reprecipitated with ten volumes of methanol. After standing for 24 hr at 4 C, the precipitate was collected by centrifugation and dried over P_2O_5 in vacuo.

The crude polysaccharide material thus obtained was partially solubilized in 100 ml of 0.02 M carbonate-bicarbonate buffer (pH 10). Both soluble and insoluble fractions were separated by centrifugation. The material solubilized at pH 10 was termed "polysaccharide I." The insoluble fraction was completely solubilized by further treatment with 100 ml of 0.02 M citrate buffer (pH 5). The material soluble at pH 5 was termed "polysaccharide II."

Both types of polysaccharides, I and II, were found in all six strains of *Nocardia* studied. They were purified by deproteinization with an equal volume of chloroform. The chloroform phase was discarded. This operation was repeated four more times. The polysaccharides in the aqueous layer

were then precipitated with methanol as above. After drying over P_2O_5, a final washing with acetone was made. The precipitate of polysaccharide I was dissolved in a NaOH solution (pH 10), whereas polysaccharide II was dissolved in a HCl solution (pH 3). Solutions of each polysaccharide were further purified by treatment with 25 ml of 5% trichloroacetic acid. The trichloroacetic acid-precipitating material was scant for polysaccharide I and significantly heavier for polysaccharide II. The trichloroacetic acid precipitates consisted mainly of protein, except for the precipitates of solutions of polysaccharide II from *N. asteroides* which contained 18 to 20% glucose.

The trichloroacetic acid supernatants were dialyzed for 72 hr against running tap water. The nondialyzable material was precipitated twice with methanol as above, and dried over P_2O_5 under reduced pressure.

For convenience, the purified polysaccharides will be abbreviated: Poly I Na, Poly II Na, Poly I Nb, and Poly II Nb, where Poly I and II stand for polysaccharides I and II, respectively, while Na and Nb stand for *N. asteroides* and *N. brasiliensis*, respectively.

Polysaccharide composition. Optimal hydrolysis of the polysaccharides was achieved with 1 N H_2SO_4 at 100 C in 6 hr (Fig. 1). Polysaccharides from all strains behaved similarly to the corresponding ones exemplified in the figure.

The monosaccharide composition of the *Nocardia* polysaccharides is given in Table 1. It can be seen that both Poly I Na and Poly I Nb contained primarily arabinose and a smaller amount of galactose. Both Poly II Na and Poly II Nb contained three monosaccharides. In addition to arabinose and galactose, they also contained mannose. Repeated trials using appropriate sprayers failed to indicate the presence of spots corresponding to uronic acids or amino sugars.

Individual sugar determinations of the different polysaccharide hydrolysates accounted for roughly 80 to 90% of their composition. Protein and phosphorus were also detected in considerable amounts. The phosphorus was probably present as phosphatides, but this possibility was not investigated. Whether the protein- or phosphorus-containing compounds, or both, are essential for the biological activities of the polysaccharides remains to be elucidated.

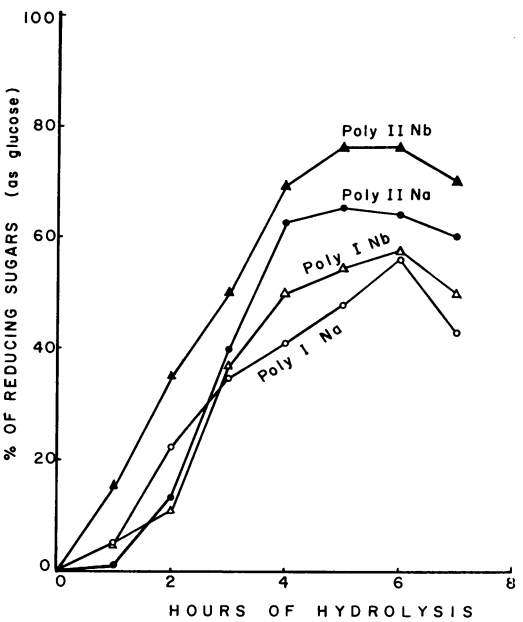

FIG. 1. *Time course of hydrolysis of purified Nocardia polysaccharides with* 1 N H_2SO_4 *at 100 C. Polysaccharides obtained from N. asteroides ISET-20 and N. brasiliensis UPHG-24.*

Although the molar ratios of the component monosaccharides never agree so closely for the same polysaccharide isolated from the three strains of either of the two nocardias studied, certain similarity was observed between Poly I Na and Poly I Nb. As will be further mentioned, this similarity was not casual; moreover, it was reinforced by immunological observations.

Both Poly II Na and Poly II Nb contained the same three individual sugars. However, the arabinose-galactose relationship was inverted in the two species. In *N. asteroides*, galactose was predominant over arabinose, whereas in *N. brasiliensis* arabinose was more abundant than galactose. In both polysaccharides, mannose was found in similar proportions (Table 1). This striking difference was also obvious in precipitin reactions.

Immunological studies. Precipitin antibodies were detected in the rabbit sera at the end of the immunization schedule; serum antibodies against *N. asteroides* polysaccharides appeared 1 week earlier than those against *N. brasiliensis*. All sera gave sharp bands of precipitation in agar at dilutions of up to 1:8. Except for the Poly II Nb system which gave two bands of precipitation, all

TABLE 1. *Chemical analysis of purified Nocardia polysaccharides**

Polysaccharide	Strain	Nitrogen	Protein	Phosphorus	Arabinose	Galactose	Mannose	Molar ratios†
		%	%	%	%	%	%	
Poly I Na	ISET-20	0.7	1.3	0.7	55	30	—	2.2:1
	ISET-1160	0.9	2.5	0.8	58	26	—	2.6:1
	UPHG-121	0.5	1.9	0.5	53	30	—	2.1:1
Poly II Na	ISET-20	0.9	3.8	0.2	22	44	12	1.2:2:1
	ISET-1160	0.8	4.9	0.1	15	54	12	1.5:4.5:1
	UPHG-121	0.9	4.1	0.2	20	50	19	1.3:2.7:1
Poly I Nb	UPHG-23	1.0	4.8	0.8	60	26	—	2.7:1
	UPHG-24	1.1	5.1	0.3	58	26	—	2.6:1
	UPHG-39	0.9	4.3	0.2	67	24	—	3:1
Poly II Nb	UPHG-23	0.9	4.6	1.0	49	28	13	4.4:2:1
	UPHG-24	1.4	6.9	0.8	48	27	13	4.3:2:1
	UPHG-39	1.0	5.9	0.1	54	19	12	5.9:1.7:1

* Except for the nitrogen and the protein determinations which were made on intact polysaccharides, all determinations were carried out on 6-hr hydrolysates. For abbreviations of the polysaccharides, see text.

† Of arabinose-galactose or arabinose-galactose-mannose.

systems gave only one band (Fig. 2). The precipitin bands appeared well-defined 72 hr after the antigens and antisera were allowed to diffuse in the agar plates. However, 24 and 48 hr were enough in reactions with Poly II Na and Poly II Nb antigens, respectively.

Poly I Na and Poly I Nb reacted indistinctly in agar diffusion tests with either *N. asteroides* or *N. brasiliensis* antisera. Moreover, both antigens absorbed serum antibodies against each other when incubated with antisera of either *Nocardia* species. It appears, then, that Poly I Na is very similar to if not identical with Poly I Nb.

A completely different picture is observed when the immunological behavior of Poly II Na and Poly II Nb is examined. They neither cross-reacted between themselves or with the Poly I's, nor did they absorb serum antibodies against each other; that is to say, they are species-specific. Representative pictures of the precipitin reactions of *Nocardia* polysaccharides are shown in Fig. 2. Reproducibility of these immunological tests was verified with different batches of the polysaccharides.

Results obtained with the serological studies just described prompted us to assess the usefulness of these reactions in the serological diagnosis of mycetoma in humans. The results obtained thus far (Bojalil and Zamora, *unpublished data*) have been most rewarding. It may be advanced that sera from patients with mycetoma due to *N. brasiliensis*, as well as patients with tuberculosis and leprosy, contained antibodies against Poly I Na (or Poly I Nb). In addition, antibodies against Poly II Nb were detected only in the sera of patients with mycetoma due to *N. brasiliensis*.

DISCUSSION

The polysaccharides I from *N. asteroides* and *N. brasiliensis* are very similar, both chemically and immunologically. They both contain arabinose and galactose in similar molar ratios (from 2:1 to 3:1). This is in comparison with an arabinose-galactose molar ratio of 1.7:1 obtained by Bishop and Blank (1958) for a polysaccharide of *N. asteroides* isolated by alkaline extraction, a method more drastic than that used by the present authors. Polysaccharide I may be the cause, or one of the causes, of the cross reactivity usually observed between *N. asteroides* and *N. brasiliensis* (González-Ochoa and Vázquez-Hoyos, 1953) and between the *Nocardia* and the mycobacteria (Schneidau and Shaffer, 1960; Cummins, 1962) in antigen-antibody reactions. In support of our assumption, earlier workers have identified polysaccharides containing primarily arabinose and galactose in the mycobacteria (Anderson and Creighton, 1939). This is also in accord with

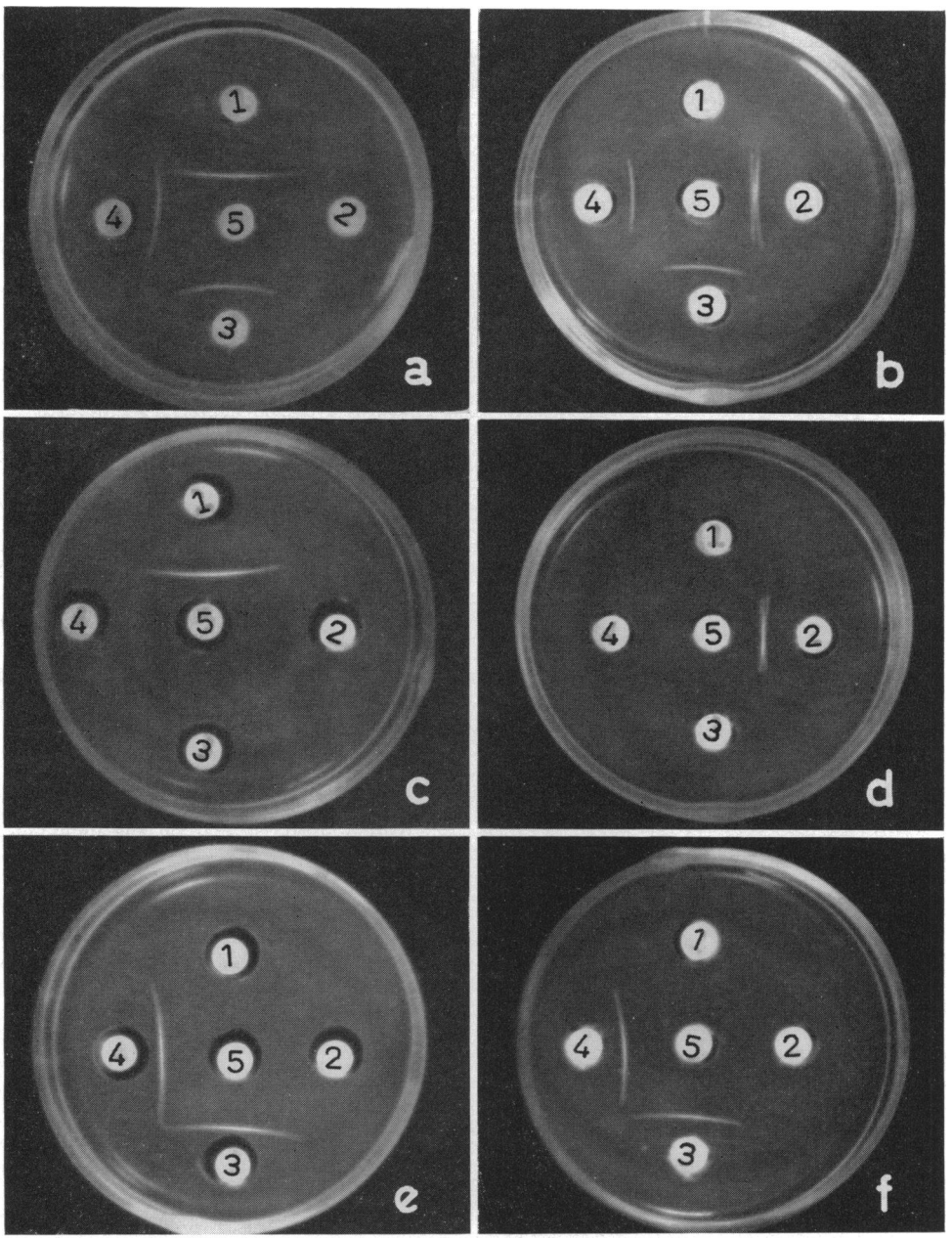

FIG. 2. *Double-diffusion precipitation reactions of antisera and polysaccharide antigens of Nocardia asteroides and N. brasiliensis. Peripheral wells of all plates contain the purified antigens:* (1) *Poly II Na;* (2) *Poly II Nb;* (3) *Poly I Na;* (4) *Poly I Nb. Center wells marked with no.* 5 *contain different antisera;* (a) *and* (b) *unabsorbed N. asteroides and N. brasiliensis antisera, respectively;* (c) *N. asteroides antiserum absorbed with Poly I Na (same result if absorbed with Poly I Na);* (d) *N. brasiliensis antiserum absorbed with Poly I Nb (same result if absorbed with Poly I Na);* (e) *N. asteroides antiserum absorbed with Poly II Na;* (f) *N. brasiliensis antiserum absorbed with Poly II Nb.*

our observations on the presence of antibodies against polysaccharides I in sera of patients afflicted with tuberculosis or leprosy (Bojalil and Zamora, *unpublished data*). Recent investigations have shown that arabinose and galactose are the main monosaccharide components of the cell wall of *Nocardia* and mycobacteria (Romano and Sohler, 1956; Földes, 1959; Kotani et al., 1959, 1960; Cummins, 1962).

The polysaccharides II isolated from *Nocardia* contained mannose in addition to arabinose and galactose. The molar ratios of these last two sugars were inverted in the two *Nocardia* species studied. They showed in common, besides their individual sugar composition, solubility characteristics. Nevertheless, they were species-specific; antibodies against these polysaccharides were evoked only by the homologous *Nocardia* species. Polysaccharides with a monosaccharide composition similar to that of the polysaccharides II from *Nocardia* have been reported only for *Mycobacterium* species (Roberts and Anderson, 1931; Haworth, Kent, and Stacey, 1948; Kwapinski and Snyder, 1961). It seems too early to ascribe to polysaccharides II a definite structural position in the cell.

ACKNOWLEDGMENT

The authors express their appreciation to Félix Córdoba, Department of Biochemistry, Faculty of Medicine, University of Mexico, for many helpful suggestions throughout this work.

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