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# Isolation of Polysaccharides from Tissues Infected with *Mycobacterium lepraemurium* [1,2]

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In a previous paper ([5]) we described the isolation of group-specific polysaccharides from tissues rich in *Mycobacterium leprae.* The group-specific polysaccharide (PolyINb), which is present in *Nocardia* and mycobacteria ([1, 3, 6, 9]), has been characterized chemically as a polymer of D-arabofuranose and D-galacto-pyranose in a molar ratio 3:1 ([4]).

The present paper deals with the identification of the group-specific polysaccharide (PolyINb) and other polysaccharides in extracts of infected tissues rich in *M. lepraemurium* from mice. Identification of polysaccharides was carried out after physical removal and enzymatic destruction of all other components that might have been immunologically active.

## MATERIALS AND METHODS

**Isolation of polysaccharides.** The Hawaii strain of *M. lepraemurium* was used. Mice (strain CFW) 120 days after inoculation were used. All tissues containing large amounts of *M. lepraemurium* were cut into pieces (100 gm. net weight) and washed with sterile physiologic saline solution. The washed tissues were homogenized in a Vir-Tis homogenizer at 2°C, suspended in saline, and adjusted to pH 2 with M HCl. Pepsin (Difco 1:10,000) was added to give a final concentration of 2.5 per cent, and the mixture was incubated at 37°C for four days, a pH of 2 being maintained with M HCl. Toluene was added to prevent bacterial growth.

The pepsin-digested suspension was neutralized with M NaOH and centrifuged at 0°C at 3,000 rpm for one hour. After this centrifugation three layers were obtained, a sediment, a liquid middle layer, and an upper layer. The latter contained a large amount of *M. lepraemurium* together with some cellular residues. The middle layer was practically free from microorganisms or cells. Acid-fast rods were present in the sediment but with a large amount of cellular residues.

Part of the upper layer was resuspended in saline and divided in two parts. One was chilled and subjected to the action of a Sonifer, S-125, at maximal intensity for 17 minutes. The other part was frozen and thawed 10 times. Both samples were then centrifuged at 0°C at 3,000 rpm for one hour; the supernatants were collected and designated crude fractions 1 and 2 respectively. One-half of each crude fraction (i.e., 1 and 2) was treated with B-ribonuclease and deoxyribonuclease I as described previously ([5]) to break down nucleic acids. The product was then dialyzed against several changes of distilled water and the material remaining was treated five times with an equal volume of chloroform-butanol 9:1 v/v ([8]) to precipitate and thus remove proteins. The resultant solutions were concentrated under reduced pressure in a rotatory evaporator and designated purified fractions 1 and 2 respectively.

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**Determination of sugars.** Sugars were estimated by the Molisch and the phenol-sulfuric acid method ([2]).

**Immunologic procedures.** Agar precipitations were carried out in 1 per cent agar (Ion agar, Oxoid) containing 0.85 per cent NaCl and Merthiolate (final concentration 100 μg/ml.) at pH 5.8-6.2. The plates were incubated for 24-72 hours either at room temperature or at 4°C ([7]).

Precipitation in capillary tubes was performed by mixing equal volumes of serum and antigen and incubating as before.

## RESULTS

The purified fractions 1 and 2 gave positive Molisch and phenol-sulfuric acid reactions. The total amounts of polysaccharide obtained, as estimated by the phenol-sulfuric acid method, were 800 and 400 μg. for purified fractions 1 and 2 respectively, a mixture of D-arabinose and D-galactose in a molar ratio 3:1 being used as a standard. After concentration in the evaporator, the final concentration of polysaccharide (in 0.15 M saline) was 100 or 200 μg/ml. Both crude fractions were also concentrated and adjusted to a final concentration of 200 μg/ml. (in 0.15 M saline) as polysaccharide.

In capillary tubes all four fractions, crude and purified, precipitated with three sera from lepromatous patients and also with a serum from a patient with active tuberculosis (designated anti-tb serum). The above named sera contained precipitins against the group-specific antigen PolyINb. The sera from 12 healthy persons failed to precipitate.

In Ouchterlony's agar plates (Fig. 1) purified fraction 1 and purified fraction 2 gave three bands with a serum from a lepromatous patient. One band appears to be immunologically identical to that for

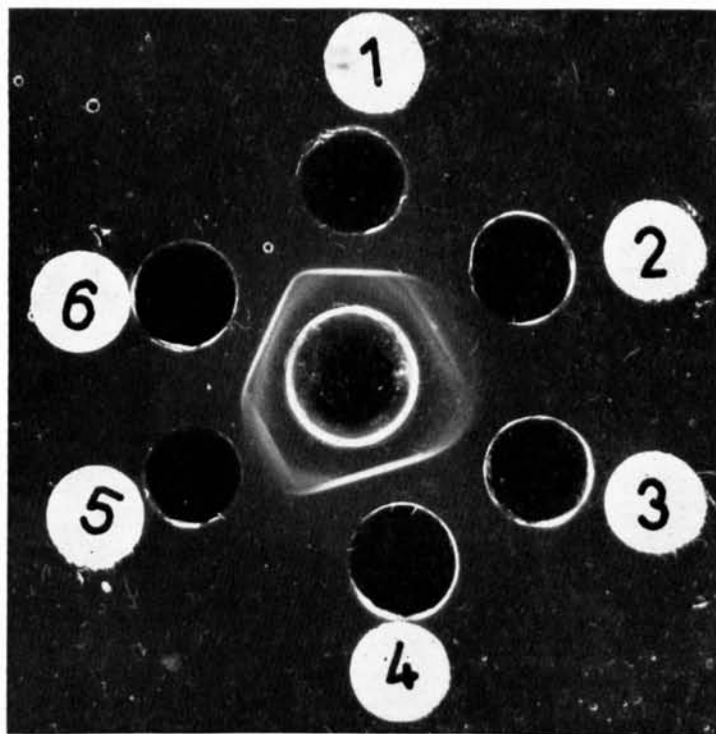


Fig. 1. **1.** Polysaccharide I from *N. brasiliensis:* 100 μg/ml. **2.** Purified polysaccharides from *M. lepraemurium* (purified fraction 1) 200 μg/ml. **3.** Purified polysaccharides from *M. leprae:* 100 μg/ml. **4.** Polysaccharide I from *N. brasiliensis:* 150 μg/ml. **5.** Purified polysaccharides from *M. lepraemurium* (purified fraction 2) 100 μg/ml. **6.** Purified polysaccharides from *M. tuberculosis:* 120 μg/ml. **Center:** Serum from a patient with lepromatous leprosy.

PolyINb, and the other two bands showed partial identity with PolyINb. In the same plate polysaccharides of *Mycobacterium tuberculosis*, obtained by mechanical rupture of the bacterial cells, gave two bands, one corresponding to PolyINb.

The anti-tb serum when absorbed with PolyINb no longer precipitated with purified fractions, and three sera from three rabbits immunized against *Nocardia brasiliensis* precipitated with all four fractions. No precipitation was found with seven other sera from lepromatous patients. In Ouchterlony's agar plates 12 sera from healthy persons failed to precipitate with all four fractions.

## DISCUSSION

After enzymatic and physical elimination of proteins and nucleic acids, immunologically active substances of polysaccharide nature remained. These polysaccharides could be obtained from preparations rich in *M. lepraemurium* either by sonication or by freezing and thawing. The scarcity of the active substances precluded determination of their constituent sugars, but their inability to dialyse was a proof of their high molecular weights.

The existence of identity bands for PolyINb and the purified fractions, and the absorption of the reacting precipitin with PolyINb obtained from *N. brasiliensis* cells, indicated the presence of an immunologically identical group antigen in *M. lepraemurium*. On the other hand *M. lepraemurium* seems to contain other polysaccharides, which gave a band of partial identity with PolyINb.

The fact that all four fractions precipitated with sera from lepromatous patients suggests an immunologic relation between *M. lepraemurium* and *M. leprae*. The band of identity between PolyINb and the extract of *M. tuberculosis* suggests the presence of an immunologically identical substance in *M. tuberculosis*.

## SUMMARY

From preparations rich in *Mycobacterium lepraemurium* three immunologically active polysaccharides have been isolated. The polysaccharides were obtained by sonication, or by freezing and thawing, of *M. lepraemurium*, and after enzymatic and physical removal of proteins and nucleic acids. One of the polysaccharides gave a band, in agar, immunologically identical with that produced by the group-specific antigen Polysaccharide I (PolyINb), isolated from *Nocardia brasiliensis*. The other polysaccharide gave a band of partial identity with PolyINb. The polysaccharide preparations precipitated with sera from rabbits immunized against *N. brasiliensis*, with sera from three lepromatous patients, and with a serum from a patient with active tuberculosis. Sera from 12 healthy donors, as well as seven sera from other lepromatous patients, failed to precipitate.

## RESUMEN

De preparaciones ricas en *Mycobacterium lepraemurium* se aislaron tres polisacáridos inmunológicamente activos. Los polisacáridos se obtuvieron por rompimiento ultrasónico, o por congela miento y descongelamiento, de *M. lepraemurium*, y después de eliminar enzimática y fisicamente tanto protéinas como acidos nucleicos.

Uno de los polisacáridos dió, en agar, una banda de precipitación de identidad con el antígeno de grupo Polisacárido I (PolyINb) aislado de *Nocardia brasiliensis*. Los otros polisacáridos dieron una banda de identidad parcial con PolyINb. La preparación de polisacáridos precipitó con sueros de conejos inmunizados con *N. brasiliensis*, con tres sueros de leprosos lepromatosos, y con el suero de un enfermo con tuberculosis activa. Los sueros de 12 personas sanas, así como sueros de otros siete pacientes lepromatosos, no precipitaron con las preparaciones de polisacáridos.

## RÉSUMÉ

A partir de preparations riches en *Mycobacterium lepraemurium* on a separe trois polysaccharides immunologiquement actifs. Les polysaccharides ont été obtenus par brisement ultrasonique ou par congélation et décongélation de *M. lepraemurium*, et aprés d'écarter enzimatique et physiquement des protéines et des acides nucleiques.

Un des polysaccharides a donné, en agar, une bande de précipitation d'identité avec l'antigene de groupe polysaccharide I (PolyINb), isolé de *Nocardia brasiliensis*. Los autres polysaccharides ont donné une bande d'identité partielle avec PolyINb. La preparation de

polysaccharides a precipitée avec des sérums de lapins immunisés avec *N. brasiliensis*, avec trois serums de lépreux lepromateux, et avec le sérum d'un malade a tuberculose active. Les serums de 12 personnes saines, aussi bien que les serums d'autres sept malades lepromateux, n'ont pas précipité avec les preparations du polysaccharides.

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