Isolation of the *Mycobacterium leprae*-Specific Glycolipid Antigen, Phenolic Glycolipid-I, from Formalin-Fixed Human Lepromatous Liver

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A *Mycobacterium leprae*-specific phenolic glycolipid antigen was purified from Formalin-fixed liver preserved from an advanced lepromatous leprosy patient. Its chemical and immunological properties were compared with those of phenolic glycolipid-I obtained from *M. leprae*-infected armadillo liver. Based on the findings that the glycolipids from the two sources have the same thin-layer chromatographic properties, infrared absorption spectrum, sugar composition, and seroreactivity, we conclude that large quantities of the phenolic glycolipid-I antigen are produced in human lepromatous leprosy lesions and that Formalin-fixed lepromatous livers and spleens from the prechemotherapeutic era are suitable sources of the glycolipid.

In 1980, Brennan and Barrow (1) reported that a partially purified lipid fraction from *Mycobacterium leprae* yielded distinct lines of precipitation with sera from lepromatous leprosy patients and *M. leprae*-infected armadillos. Based on the findings that the activity was unaffected by mild alkali and that the fraction yielded 6-deoxyhexoses upon hydrolysis, it was suggested that the lipid antigens may be related to the phenolphthiocerol-containing lipids of *Mycobacterium* strains variously called the A, B, or G mycosides. Hunter and Brennan (5) isolated large quantities of the glycolipids from *M. leprae*-infected armadillo liver residue that was free of the bacillus. The molecular structures of the glycolipids have been fully elucidated (4-7) and named phenolic glycolipid-I (PGL-I), PGL-II, and PGL-III. They are all phenolic-diacylphthiocerol triglycosides in which the trisaccharide portion is unique to the leprosy bacillus. Since the PGLs are strongly antigenic and highly specific for *M. leprae*, they have received considerable favor as probes for the serodiagnosis of leprosy (2, 3, 12), are thought to modulate the selective immunological unresponsiveness characteristic of lepromatous leprosy (9), and may be implicated in the intracellular persistence of the bacillus (6). Nevertheless, there has been no direct evidence to demonstrate that the PGLs as such are produced in human leprosy lesions.

In this paper, we describe the isolation of PGL-I from Formalin-fixed lepromatous liver and conclude that, as was discovered in experimentally infected armadillos, the glycolipid is produced in large quantities in human lepromatous tissue.

Formalin-fixed liver slices (85 g) from an advanced lepromatous patient who had died of laryngeal leprosy were lyophilized for 4 h to remove Formalin and water. The dried specimen (19.5 g) was homogenized in 800 ml of chloroform-methanol (2:1) in an ultrahigh-speed homogenizer (Polytron; Kinematica GmbH, Switzerland) and extracted at 50°C for 18 h. The tissue residue was suspended in 300 ml of chloroform-methanol (2:1) and again incubated at 50°C for 18

h. The dried lipids (2.624 g) were mixed with ether, and the ether-insoluble fractions were removed by centrifugation at $3,000 \times g$ for 20 min.

Ether-soluble lipids (2.221 g) were dissolved in a minimal volume of chloroform and applied to a column (16 by 2 cm) of Florisil (100/200 mesh, Floridin Co., New York). The column was developed with 2 bed volumes each of chloroform, 2% methanol, and 5% methanol in chloroform. Eluates were monitored for the presence of PGL-I by silica gel thin-layer chromatography (Alumgram Sil G, Federal Republic of Germany) in chloroform-methanol-water (45:5:0.5) alongside the authentic material derived from infected armadillos. The glycolipid-containing fractions in the 2 and 5% methanol eluates were collected, concentrated, and fractionated on a column (10 by 1 cm) of silicic acid (100 mesh; Mallinckrodt Inc., Paris, France). The 2% methanol-chloroform eluates contained the pure glycolipid (Fig. 1). The glycolipids from the two sources, human and armadillo, showed similar mobilities ($R_f = 0.48$) and color reactions (Fig. 1). The infrared absorption spectra of both were identical (Fig. 2).

For analysis of the constituent sugars, the pure glycolipid (500 μ g) was hydrolyzed with 2 M CF_3COOH and converted to the alditol acetates by reduction with sodium borohydride, followed by acetylation with acetic anhydride (5). Gas-liquid chromatography of the alditol acetates showed the presence of the acetates of 2,3-di-*O*-methyl-rhamnitol, 3-*O*-methyl-rhamnitol, and 3,6-di-*O*-methyl-glucitol in approximately equimolar amounts (Fig. 3). These were positively identified through cochromatography with the alditol acetates derived from PGL-I from infected armadillo organs and by combined gas chromatography-mass spectrometry. The mass spectrum of the 2,3-di-*O*-methyl-rhamnitol acetate showed major fragments at m/z 101, 117, 143, 161, and 203 and thus corresponded to that of the authentic sugar derivative (5, 8). The 3-*O*-methyl-rhamnitol acetate showed major peaks at m/z 87, 101, 129, 143, 189, and 203, and thus the spectrum was that of a 3-*O*-methyl-6-deoxyhexitol (5, 8). Likewise, the spectrum of the 3,6-di-*O*-methyl-glucitol acetate was identical to that of a 3,6-di-*O*-methyl-hexitol, with

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FIG. 1. Silica gel thin-layer chromatography of PGL-I from *M. leprae*-infected armadillo liver (A) and Formalin-fixed liver of a lepromatous leprosy patient (H). Chromatography was conducted in chloroform-methanol-water (45:5:0.5), and color was developed by spraying with 1% orcinol in 40% H_2SO_4 and charring at 120°C for 3 min.

major fragments at m/z 87, 129, 189, and 233 (5, 8). The evidence of thin-layer chromatography and infrared homology combined with an identical sugar profile strongly indicates that the products from the human and armadillo sources are identical.

The antigenicity of the PGL-I from lepromatous liver was tested against human leprosy sera in an enzyme-linked immunosorbent assay designed to accommodate the extreme lipidity of the antigen. Sera were collected in the outpatient clinic of the Leprosy Research Laboratory, Kyoto University, Kyoto, Japan, from patients with lepromatous, borderline, and tuberculoid leprosy. The lepromatous patients were both polar lepromatous and borderline lepromatous types, and the borderline group contained both borderline and borderline tuberculoid cases, as described by the Ridley-Jopling classification (10). Ten sera from patients with other mycobacterial diseases, including nine pulmonary tuberculosis cases and one case with atypical mycobacterial infection, were collected in the Chest Disease Research Institute, Kyoto University, and the National Toneyama Hospital, Osaka, Japan.

FIG. 2. Infrared absorption spectra of PGL-I from *M. leprae*-infected armadillo liver and Formalin-fixed human liver.

FIG. 3. Gas-liquid chromatography of the alditol acetates of the sugars of PGL-I from the human source. The conditions were as described by Hunter and Brennan (4). A column (180 by 0.2 cm) of 3% SP-2340 on 100/120-mesh Supelcoport operating isothermally at 190°C was used.

An ethanol solution of the human PGL-I (4 $\mu g/ml$; 50 μl) was added to the wells of polystyrene enzyme-linked immunosorbent assay plates (Immuno Plate II; Nunc, Kamstrup, Denmark), and the solvent was evaporated by heating at 60°C. Two-thirds of the wells were coated with PGL-I, and the rest contained ethanol only. The dried wells were each blocked by incubating at 37°C for 60 min with 100 μl of 5% bovine serum albumin. After removal of the bovine serum albumin, 50 μl of test serum (diluted 20 times with 20% fetal calf serum-phosphate-buffered saline) was added, and the plates were incubated at 37°C for 60 min. Wells were washed four times in 0.05% Tween 20-phosphate-buffered saline with Nunc Immuno Wash 12. Peroxidase-conjugated anti-human immunoglobulin (G + A + M) antibody (Dako-Immunoglobulins a/s, Denmark) (50 μl), diluted with 20% FCS-phosphate-buffered saline, was added to each well and incubated at 37°C for 60 min. After removing excess conjugate with 0.05% Tween-phosphate-buffered saline, 100 μl of substrate solution (*o*-phenylenediamine [0.4 mg/ml], 30% H_2O_2 [0.4 $\mu l/ml$] in citrate-phosphate buffer [pH 5.0]) was added, plates were developed in the dark for 15 to 30 min, and the reaction was stopped by the addition of 100 μl of 2.5 N H_2SO_4 . The optical density was measured with a two-wavelength microplate photometer (MTP-22; Corona Electric, Japan) at wavelengths of 492 and 610 nm. The results of the enzyme-linked immunosorbent assay are summarized in Table 1. Fifty-four of 68 (79%) sera from lepromatous patients, 8 of 13 (62%) borderline sera, and 5 of 13 (39%) tuberculoid sera were positive. All 10 sera from patients with other mycobacterioses, i.e., 9 cases of pulmonary tubercu-

TABLE 1. Estimation of anti-PGL-I antibodies in sera from patients with leprosy and other mycobacterioses, with PGL-I from the human source^a

Disease type	Total no. of sera	No. of sera showing indicated reaction		% Positive ^b
		Positive	Negative	
Lepromatous	68	54	14	79
Borderline	13	8	5	62
Tuberculoid	13	5	8	39
Other mycobacterial diseases	10	0	10	0

^a Enzyme-linked immunosorbent assay conditions are described in text.

^b Positive, >3 standard deviations from the mean of the healthy controls.

losis and 1 case with atypical mycobacterial infection, were negative. Thus, the seropositivity rate with the human product is similar to that recorded with the armadillo-derived glycolipid (3).

PGL-I, PGL-II, and PGL-III are unique antigenic glycolipids synthesized exclusively by *M. leprae*. PGL-I is the major component. The molecular structure (5) is as follows: 3,6-di-*O*-methyl-Glcp(β1→4)2,3-di-*O*-Me-Rhap(α1→2)3-*O*-Me-Rhap 1α→phenolphthiocerol dimycocerosate. PGL-II and PGL-III are minor metabolic by-products of PGL-I.

The present study demonstrates that the glycolipids from the human and armadillo sources correspond according to several parameters, viz., thin-layer chromatography mobility, infrared absorption, sugar composition, and seroreactivity, and thus clearly indicates that PGL-I is also synthesized in human lepromatous lesions. Young (11) had reported that lipid extracts from human lepromatous skin biopsies contained a 6-deoxyhexose-containing glycolipid, and suggested that the responsible glycolipid may be PGL-I. The present study shows the veracity of this contention.

This study also indicates that Formalin-fixed human lepromatous liver is a highly suitable source of *M. leprae* antigens. Since large stocks of heavily infected, Formalin-fixed livers and spleens are present in the national leprosy hospitals of Japan and other countries, mostly arising from the prechemotherapeutic era, these should provide an alter-

native source to infected armadillos for the provision of the specific PGL.

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