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CHEMICAL SYNTHESIS AND SEROREACTIVITY OF *O*-(3,6-DI-*O*-METHYL-β-D-GLUCOPYRANOSYL)-(1→4)-*O*-(2,3-DI-*O*-METHYL-α-L-RHAMNOPYRANOSYL)-(1→9)-OXYNONANOYL-BOVINE SERUM ALBUMIN—THE LEPROSY-SPECIFIC, NATURAL DISACCHARIDE-OCTYL-NEOGLYCOPROTEIN

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ABSTRACT

The outer disaccharide segment, namely, *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranose, of the trisaccharide-containing, leprosy-specific, phenolic glycolipid I has been synthesized as the 8-(methoxycarbonyl)octyl glycoside in high yield and absolute stereospecificity by a series of modified Koenigs–Knorr and Helferich reactions. A particular feature of the synthetic pathway involves methylation of the 2-hydroxyl group of the rhamnose moiety under neutral conditions, after first preparing the 8-(methoxycarbonyl)octyl glycoside as the α anomer *via* the 1,2-orthoacetate, and thus precluding the possible formation of an anomeric mixture. The 8-(methoxycarbonyl)octyl *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranoside was converted into the crystalline hydrazide, and this was coupled to bovine serum albumin (BSA), *via* intermediate acyl-azide formation, to produce the corresponding neoglycoprotein, *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-α-L-rhamnopyranosyl)-(1→9)-oxynonanoyl-BSA, the so-called natural disaccharide-octyl-BSA. Extensive serological testing of this product against sera from leprosy patients and control populations, and comparison with the native glycolipid and previously synthesized neoglycoproteins, have shown that it is unparalleled in terms of sensitivity and specificity, and highly suited to replace the native glycolipid for the serodiagnosis of worldwide lepromatous leprosy.

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INTRODUCTION

The recent isolation and complete structural elucidation[1–3] of a *Mycobacterium leprae*-specific antigen, the so-called phenolic glycolipid I (PGL-I), has contributed immensely towards the specific serodiagnosis of leprosy[4–6], and now provides the means for identification of active, lepromatous leprosy cases. PGL-I is the trisaccharide glycoside of the *p*-substituted phenol phthiocerol, which possesses a branched glycol chain, the hydroxyl groups of which are esterified with branched-chain fatty acids called mycocerosic acids. The trisaccharide unit is unique to the leprosy bacillus. The exact structure of PGL-I is: 29-{4-[*O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-α-L-rhamnopyranosyl)-(1→2)-3-*O*-methyl-α-L-rhamnopyranosyloxy]phenyl}-3-methoxy-4-methyl-9,11-non-acosanediol 9,11-dimycocerosate[3].

By examining the capacity of PGL-I and its various partially deglycosylated degradation products[3] to bind to polyclonal immunoglobulin M (IgM) antibodies from leprosy patients[7] and murine monoclonal IgM[8] and immunoglobulin G (IgG)[9] antibodies, it was clearly demonstrated that all of the antibody-binding capacity is resident in the terminal disaccharide, and primarily in the nonreducing 3,6-di-*O*-methyl-β-D-glucopyranosyl unit. The trisaccharide itself[7,10], the terminal disaccharide[7,11,12], and various incompletely *O*-methylated analogs have been synthesized, and, in antibody-inhibition assays, they showed exquisite specificity for both the 3- and 6-*O*-methyl substituents[7]. Likewise, neoglycoproteins prepared by reductive amination with the oligosaccharides[13] demonstrated the need for full *O*-methylation and an intact glycosyl-ring structure of correct anomeric configuration.

Despite the success of some hitherto synthesized 3,6-di-*O*-methyl-D-glucopyranose-containing neoglycoproteins in recognizing the majority of anti-glycolipid IgM antibodies in patient sera, they showed false-positive reactions with some normal sera and with those from high-responder tuberculosis patients[13,14]. Yet, neoglycoproteins are essential for the control of worldwide leprosy; the glycolipid itself, derived from the experimentally infected aramadillo, is very expensive and laborious to prepare[15]. We now describe the synthesis of the disaccharide epitope of phenolic glycolipid I, conjugated *via* an 8-(methoxycarbonyl)octyl linker-arm[16,17] to BSA, the so-called natural disaccharide-octyl-BSA (ND-O-BSA), and demonstrate by serological comparison with other neoglycoproteins that it best emulates the natural antigen in sensitivity and specificity.

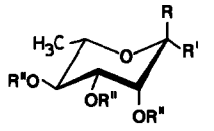
RESULTS AND DISCUSSION

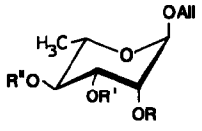
Synthesis of the natural disaccharide-octyl-BSA (ND-O-BSA). — The recent successful application, to leprosy serodiagnosis, of neoglycoproteins based on the disaccharide-alditol[13] and on the terminal 3,6-di-*O*-methyl-β-D-glucopyranoside linked *via* an 8-carbonyloctyl bridge[14] stimulated the present synthetic program.

The approach was designed to achieve, through unambiguous stereospecific synthesis, greater serological sensitivity and specificity than described heretofore. The strategy of the present synthetic program exploits the extensive work of Lemieux *et al.*[16,17] on the blood-group antigens, and of Bundle and co-workers[18] on the O-antigen determinants of *Shigella flexneri*. The use of Lemieux's "9-carbon spacer-arm" as a bridge between hapten and protein is known to provide neoglycoproteins with near-maximum substitution of haptens[17].

The synthetic methods were chosen to ensure that the ester function of the linker arm was retained throughout, and, accordingly, conditions were designed for compatibility with this feature. Thus, for instance, strong alkaline conditions were avoided. In addition, in earlier syntheses[7], the presence of a methyl substituent on O-2 of the internal rhamnosyl residue (2,3-di-*O*-methylrhamnopyranose) led to the formation of an anomeric mixture, resulting in laborious column purifications. The present pathway was designed to avoid glycosylation in the presence of the nonparticipating neighboring-group. The final yields were greater than heretofore, and one of the intermediates formed may now be utilized for synthesis of the natural trisaccharide-octyl-BSA.

The intitial stage of the synthesis required a rhamnopyranosyl unit selectively blocked at O-4. The sequence followed was essentially that used in the synthesis of the *Shigella flexneri* O-antigen determinant[18]. Thus, allyl α-L-rhamnopyranoside (**4**) was synthesized by acylation of L-rhamnose to give **1**, followed by bromination at C-1 to give **2**, and condensation of **2** with allyl alcohol in the presence of mercuric cyanide in dichloromethane, to give allyl 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranoside (**3**); this was deacylated, and **4** was converted into **5** by treatment with 2,2-dimethoxypropane in the presence of *p*-toluenesulfonic acid. Benzylation of **5** by Brimacombe's[19] procedure gave allyl 4-*O*-benzyl-2,3-*O*-isopropylidene-α-L-rhamnopyranoside (**6**) in 81% yield. Compound **6** was hydrolyzed at 0° by using 99% trifluoroacetic acid, to give **7**, and this was followed by removal of the allyl group with Wilkinson's catalyst[20]; subsequent hydrolysis furnished **8** as a crystalline compound after column chromatography. Acetylation of **8** gave **9** as a syrup.





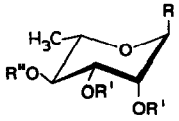
	R"	R'	R
1	Ac	H,OAc	
2	Ac	H	Br
3	Ac	H	OAll
4	H	H	OAll

All = $H_2C{=}CHCH_2-$

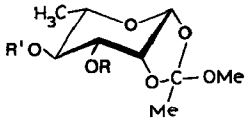
	R"	R'	R
5	H	$-CMe_2-$	
6	Bn	$-CMe_2-$	
7	Bn	H	H

Bn = $PhCH_2-$

Owing to the lability of benzyl ethers to hydrogen bromide–acetic acid, 1,2,3-tri-*O*-acetyl-4-*O*-benzyl-L-rhamnopyranose (**9**) was converted into its 1-bromide (**10**) by using trimethylsilyl bromide[21] at −10°; according to n.m.r. data, compound **10** was obtained in ~75% yield. Some starting material (**9**) remained unreacted, but, due to the lability of the halide, no attempts were made to isolate it. In order to introduce a methyl group at O-3 while having the 1,2 site blocked, the bromide **10** was converted almost quantitatively into its 1,2-orthoacetate (**11**) by using anhydrous methanol. Compound **11** was deacylated with anhydrous methanol saturated with anhydrous ammonia; 4-*O*-benzyl-1,2-*O*-(1-methoxyethylidene)-β-L-rhamnopyranose (**12**) was obtained in good yield after 24 h. Methylation of **12** with sodium hydride and methyl iodide gave **13**; dry-column chromatography, using alumina to remove impurities from reagents, gave pure **13**. The sequence **8** → **9** → **10** → **11** → **12** → **13** did not involve any fractionation, and the overall yield was 70% based on **9**.



	R"	R'	R
8	Bn	H	OH
9	Bn	Ac	OAc
10	Bn	Ac	Br

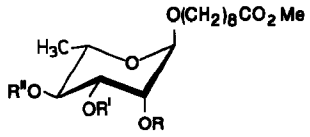


	R'	R
11	Bn	Ac
12	Bn	H
13	Bn	Me

The application of standard, orthoester glycosylation conditions[22] to **13** gave anomerically pure **14**. A longer period of heating under reflux than reported was required in order to achieve complete reaction. Compound **14** was *O*-deacetylated by conventional methods; it was observed that deacetylation in the presence of the linker arm proceeds satisfactorily if a catalytic amount of sodium methoxide (anhydrous powder) is added to the reaction mixture at 0°, and this is then brought to room temperature after 2 h, and stirred overnight at room temperature.

Compound **15** is also suitable as a glycosyl acceptor for future synthesis of the trisaccharide-containing neoglycoprotein. For present purposes, methylation at OH-2, only, was required. Conventional methylation conditions cannot be used, due to the presence of the ester function on the linker arm. Methylation employing diazomethane–BF_3 etherate had to be repeated several times in order to complete the reaction, and **16** was obtained in only 60% yield. However, methylation with methyl trifluoromethanesulfonate in the presence of 2,6-di-*tert*-butylpyridine[23] gave **16** in 80% yield after column chromatography. Compound **16** was *O*-debenzylated, to give **17** in quantitative yield.

The glycosyl donor 2,4-di-*O*-acetyl-3,6-di-*O*-methyl-α-D-glucopyranosyl bromide (**18**), whose synthesis has already been reported[14], was, due to its



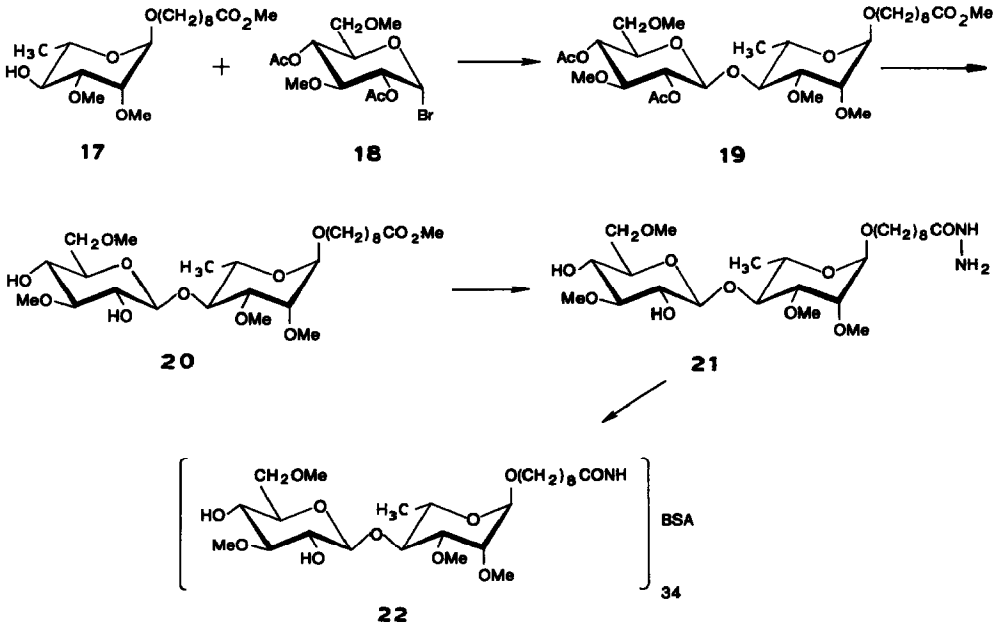
	R"	R'	R
14	Bn	Me	Ac
15	Bn	Me	H
16	Bn	Me	Me
17	H	Me	Me

instability, generated just prior to execution of the coupling reaction. Modified Helferich[24] conditions, using 5:2 mercuric cyanide–mercuric bromide in anhydrous dichloromethane, gave exclusively the desired β-linked disaccharide (**19**) in 70% yield. An excess of the catalyst (2.5 molar) led, however, to complete hydrolysis of the halide, and only traces of the disaccharide were formed. When an equimolar quantity of the "catalyst" (total mercuric salt concentration to the alcohol concentration) was used, the reaction proceeded satisfactorily. *O*-Deacylation of **19**, to give **20**, followed by conversion of compound **20** into its hydrazide by using hydrazine hydrate gave **21**, which was then made hydrazine-free. From an examination of the ^{1}H- and ^{13}C-n.m.r. spectra of **20** and the ^{13}C-n.m.r. spectrum of the hydrazide **21** in light of those of the natural[3] and synthetic[12] oligosaccharides, it was obvious that a compound having the correct structure had been obtained. Hydrolysis[3] of **19**, g.l.c.–m.s. analysis of the alditol acetates therefrom on 3% of SP-2340, and comparison with authentic products showed the presence of a 1,4,5-tri-*O*-acetyl-6-deoxy-2,3-di-*O*-methylhexitol (*m/z* 203, 161, 143, 117, and 101) and 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylglucitol (*m/z* 233, 189, 129, 113, and 87).

The hydrazide **21** was converted into its acyl azide, and this, in turn, was conjugated to the carrier protein (BSA) giving **22**. The final neoglycoconjugate preparation containing the ND-O-BSA was dialyzed, and subjected to gel filtration on Sephadex G-75. The void volume was analyzed for hapten content, divided into 0.5-mL aliquots, lyophilized, and stored frozen prior to serological analyses and shipment to leprosy-endemic areas.

Analysis of the product of the first attempt at conjugation showed 13 mol of sugar hapten per mol of BSA. By varying the conditions in order to decrease the proportion of *N,N*-dimethylformamide and reagents in the acylazide-generating mixture, the proportion of hapten in a second batch was increased to 34 mol per mol of BSA. When adjusted for differing carbohydrate content, the activity of both preparations of ND-O-BSA against pooled lepromatous-leprosy serum was comparable (see Fig. 1).

Comparative serological activity of ND-O-BSA and other synthetic antigens against leprosy and other sera. — The structures of those neoglycoproteins



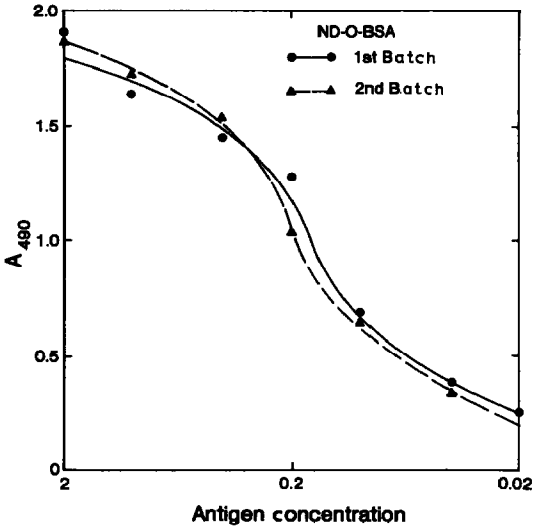
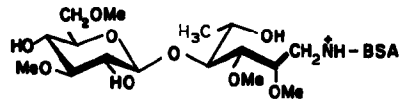
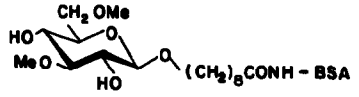


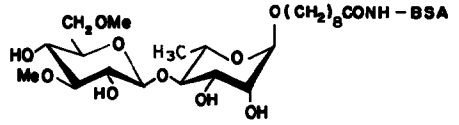
Fig. 1. Reaction of the two preparations of ND-O-BSA at decreasing concentrations against pooled, human lepromatous-leprosy serum. Concentrations of the neoglycoprotein are expressed as nmol of disaccharide equivalent. ELISA conditions are described in the Experimental section. Absorbances were read at 490 nm with a Dynatech MR600 Microplate reader.



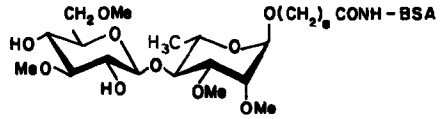
Reduced disaccharide-BSA (rD-BSA)



Monosaccharide-octyl-BSA(M-O-BSA)



Disaccharide-octyl-BSA (D-O-BSA)



Natural disaccharide-octyl-BSA(ND-O-BSA)

Fig. 2. Structures and trivial names of the leprosy-specific neoglycoproteins prepared to data and tested comparatively in the present work.

synthesized to date are presented in Fig. 2. The rD-BSA, M-O-BSA, and D-O-BSA all proved to be highly reactive against lepromatous-leprosy serum, and showed good concordance with the native glycolipid in analysis of serum specimens of individual leprosy patients; the correlation coefficient (r) values in ELISA for PGL-I and those neoglycoproteins were from 0.753 to 0.909 ($p < 0.001$, student t test)[13,14]. Despite good agreement between the native glycolipid and these earlier glycoconjugates in determining seropositivity or seronegativity, there was evidence of discrepancy. Of 223 serum specimens assayed, nine (4.0%) were positive to PGL-I, but negative to rD-BSA[13]. Furthermore, in more than 15 samples, the reactivity against rD-BSA was less than half of that against PGL-I, indicating, perhaps, that more of the native structure is required for optimal reaction with anti-PGL-I antibodies. Hence, the need for the present neoglycoprotein, the ND-O-BSA, and that described previously[14], the D-O-BSA.

D-O-BSA and ND-O-BSA were compared in ability to detect anti-PGL-I antibodies in serum specimens from leprosy patients and control groups using

TABLE I

ANTI-GLYCOLIPID IgM ACTIVITY[a] IN SERA FROM LEPROSY PATIENTS AT THE NORTHERN CALIFORNIA CLINIC, USING THE TWO DISACCHARIDE-BASED NEOGLYCOPROTEINS

Patient classification[b]	*No. of sera assayed*	*ND-O-BSA*[c]		*D-O-BSA*[d]	
		No. positive[e] *(%)*	*Mean ±SD A_{490}*	*No. positive*[f] *(%)*	*Mean ±SD A_{490}*
TT and BT	25	9 (36)	0.104 ±0.092	9 (36)	0.151 ±0.204
BB	4	2 (50)	0.383 ±0.443	2 (50)	0.367 ±0.470
LL and BL					
<2 yr of Dapsone therapy	41	38 (93)	0.715 ±0.524	36 (88)	0.571 ±0.502
>2 yr of Dapsone therapy	52	40 (77)	0.443 ±0.456	39 (75)	0.450 ±0.486

[a]Details of ELISA conditions are described in the text. [b]According to the Ridley–Jopling classification scheme: TT, polar tuberculoid; BT, borderline tuberculoid; BB, borderline; LL, lepromatous leprosy; BL, borderline lepromatous. [c]*O*-(3,6-Di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-α-L-rhamnopyranosyl)-(1→9)-oxynonanoyl-BSA. [d]*O*-(3,6-Di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-*O*-α-L-rhamnopyranosyl-(1→9)-oxynonanoyl-BSA. [e]Positive: $A_{490} \geq 0.120$. [f]Positive: $A_{490} \geq 0.150$.

ELISA, and the results were compared to those using PGL-I and the previously synthesized glycoconjugates.

The ELISA protocol described previously was employed with minor modifications. The wetting reagent, Tween 80, was included in washing solution and serum, and in conjugate diluent, at a concentration of 0.1% (v/v). For each serum specimen, the absorbance in triplicate wells with BSA only was subtracted from that with D-O-BSA or ND-O-BSA before analysis was made. The working concentration of D-O-BSA was 500–600 ng/mL of rhamnose equivalent, and 45 ng/mL for ND-O-BSA. Both D-O-BSA and ND-O-BSA gave about the same absorbance values against a lepromatous-pooled serum.

Two sets of serum specimens were assayed; one from the U.S. Public Health Service Hansen's Disease Clinic, Seton Medical Center (Daly City, California), and the other from the Hansen's Disease Clinic of the Angeles County–University of Southern California Medical Center (Los Angeles, California). The results from the first study with 122 samples, from the Seton Medical Center, are shown in Table I. There was good concordance between ND-O-BSA and D-O-BSA in seropositivity and mean absorbance among patient groups. However, ND-O-BSA showed slightly higher seropositivity than D-O-BSA; 89 (73%) by ND-O-BSA, and 86 (70%) by D-O-BSA. High absorbance to ND-O-BSA, but moderate or low to D-O-BSA, in five samples contributed to the difference in the mean absorbance; 0.715 with ND-O-BSA, and 0.571 to D-O-BSA.

The study with the second group of leprosy patients showed results similar to those for the first (see Table II). ND-O-BSA gave slightly higher seropositivity than D-O-BSA; 66 (70%) of 94 specimens assayed were positive to ND-O-BSA, and 62 (66%) to D-O-BSA. From both studies, however, it was consistently noticed that patients at the tuberculoid end of the leprosy spectrum had less anti-PGL-I antibody level than those at the lepromatous end, an observation first made with the glycolipid itself[4–6]. Among the lepromatous groups, patients with active disease, or those treated for less than two years with Dapsone, showed much higher anti-PGL-I antibodies than those with inactive disease or prolonged treatment of more than two years.

Although both ND-O-BSA and D-O-BSA showed excellent ability to react with anti-PGL-I antibodies, the slight advantage of ND-O-BSA was further demonstrated from a study of controls consisting of healthy persons, tuberculosis patients, and non-tuberculous mycobacterioses patients (see Table III). Among 223 serum specimens, five (2.2%) were positive to ND-O-BSA, and nine (4.0%) to D-O-BSA. These data may indicate that ND-O-BSA has more discriminating ability, *i.e.*, higher specificity. Thus, ND-O-BSA had a slight advantage over D-O-BSA both in sensitivity and specificity.

It proved particularly worth while to examine some of the discrepant sera against the range of available antigens. An analysis of some 16 aberrant sera allowed classification of the response into three categories (see Table IV).

The first three serum specimens (G559, G560, and TR751), that form Category

TABLE II

ANTI-GLYCOLIPID IgM ACTIVITY IN SERA FROM LEPROSY PATIENTS AT THE SOUTHERN CALIFORNIA CLINIC, USING THE TWO DISACCHARIDE-BASED NEOGLYCOPROTEINS[a]

Patient classification[b]	*No. of sera assayed*	*ND-O-BSA*		*D-O-BSA*	
		No. positive (%)	*Mean ±SD A_{490}*	*No. positive (%)*	*Mean ±SD A_{490}*
TT active	17	5 (29)	0.165 ±0.240	5 (29)	0.177 ±0.250
TT inactive	8	2 (25)	0.075 ±0.079	2 (25)	0.081 ±0.082
BL	6	5 (83)	0.526 ±0.366	4 (67)	0.525 ±0.536
LL active	14	14 (100)	1.433 ±0.650	14 (100)	1.301 ±0.721
LL inactive	18	11 (61)	0.277 ±0.258	11 (61)	0.270 ±0.248
ENL	16	15 (94)	0.867 ±0.719	13 (81)	0.832 ±0.821
Lucio reaction	6	6 (100)	1.307 ±0.666	6 (100)	1.389 ±0.533
Reversal reaction	9	8 (89)	1.067 ±0.788	7 (78)	1.024 ±0.832

[a]Conditions and positivity parameters are those described in Table I. [b]The patient-classification system, slightly different from that in Table I, has been described.

TABLE III

ANTI-GLYCOLIPID IgM ACTIVITY IN SERA FROM NORMAL INDIVIDUALS, OR THOSE WITH MYCOBACTERIOSES OTHER THAN LEPROSY, USING THE TWO DISACCHARIDE-BASED NEOGLYCOPROTEINS[a]

Patient classification	*No. of sera assayed*	*ND-O-BSA*		*D-O-BSA*	
		No. positive (%)	*Mean ±SD A_{490}*	*No. positive (%)*	*Mean ±SD A_{490}*
Healthy controls	142	4 (2.4)	0.014 ±0.027	5 (3.5)	0.026 ±0.033
Tuberculosis	66	1 (1.5)	0.029 ±0.037	4 (6.1)	0.038 ±0.043
Non-tuberculosis mycobacterioses	15	0 (0)	0.011 ±0.013	0 (0)	0.029 ±0.022

[a]Conditions and positivity parameters are those described in Table I.

TABLE IV

ANTI-GLYCOLIPID IgM ACTIVITY IN "ABERRANT" LEPROSY SERA, USING GLYCOLIPID AND NEOGLYCOPROTEINS

Category group	*Serum No.*	*Antigen*			
		PGL-I	*ND-O-BSA*	*D-O-BSA*	*M-O-BGG*
		A_{490}			
I	G559	0.142	0.048	0.032	0.065
	G560	0.125	0.028	0.055	0.053
	TR751	0.183	0.000	0.004	0.063
II	G050	0.060	0.318	0.283	0.196
	G545	0.054	0.181	0.226	0.184
	G579	0.088	0.210	0.192	0.118
III	G530	0.193	0.442	0.056	0.134
	TR752	0.516	0.552	0.080	0.079
	TR770	0.764	0.311	0.066	0.191
	G583	2.000	1.299	0.107	0.094

I, showed some activity against PGL-I but no appreciable reactivity against any of the neoglycoproteins. It would appear, therefore, that these sera contain antibodies directed to the reducing 3-*O*-methylrhamnose terminus or some component of the diacylphenolic phthiocerol. Perhaps, for optimal binding of antibodies in such sera, more of the structure of the native glycolipid needs to be emulated. Another three specimens (G050, G545, and G579) (Category II) showed the opposite trend. They demonstrated little recognition of PGL-I, but were positive to the glycoconjugates, perhaps demonstrating the advantage of introducing larger numbers of immunodeterminants. Specimens G530, TR752, TR770, and G583 (Category III) demonstrate the importance of the full penultimate sugar, 2,3-di-*O*-methylrhamnopyranose, for optimum binding to all anti-PGL-I antibodies; the ND-O-BSA showed reactivity comparable to that of PGL-I, but D-O-BSA and M-O-BGG showed negligible or very low reactivity. In particular, a dramatic difference in response was seen in specimen G583, giving further support to the evidence for the need of the second, and, possibly, the third, sugar for recognition of all glycolipid-directed antibodies in human-leprosy sera.

EXPERIMENTAL

General methods. — All solvents and reagents were purified and dried according to standard procedures[25]. Molecular sieves 4A (8–12 mesh; Sigma Chemical Company, St. Louis, MO) were dried overnight at 160° prior to use. Sodium hydride, purchased as a 60% dispersion in mineral oil (Aldrich Chemical Company, Milwaukee, WI) was washed several times with petroleum ether before use. T.l.c. was performed on aluminum plates coated with Silica Gel 60F-254 (E.

Merck, Darmstadt, Germany); zones were detected by spraying the plates with 10% (v/v) H_2SO_4 solution, with subsequent heating. For column chromatography, loading was in the range of one part of sample to 30–50 of Kieselgel 60 (60–200 mesh; American Scientific Company, Denver, CO). To develop chromatograms, distilled solvents were used at all times. Dry-column chromatography was performed by using Florisil after prior elution with the least polar of the planned solvents.

Reported melting points are uncorrected. Optical rotations were measured at 20° with a Perkin–Elmer 241 polarimeter at the sodium D-line. N.m.r. spectra were recorded with a Bruker WH 200, or a Bruker WH 270, instrument for solutions in chloroform-*d* or methanol-d_4, with tetramethylsilane as the internal standard. The proportion of haptens in neoglycoproteins was determined as described previously[13].

The following chromatographic-solvent systems were used: *A*, chloroform; *B*, 4:1 chloroform–ether; *C*, 7:1 chloroform–ether; *D*, 4:1 chloroform–methanol; *E*, 3:1 dichloromethane–acetone; *F*, 4:1 benzene–acetone; *G*, 1:1 petroleum ether–ethyl acetate; *H*, 3:2 petroleum ether–ethyl acetate; *I*, 3:1 petroleum ether–ethyl acetate; *J*, 1:1 petroleum ether–ether; and *K*, 6:2:1 ethyl acetate–methanol–water.

Allyl α-L-rhamnopyranoside (**4**). — Crystalline 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl bromide (**2**, from 1,2,3,4-tetra-*O*-acetyl-L-rhamnopyranose[26]; 38 g, 107.6 mmol) was gradually added to a mixture of dry allyl alcohol (15 mL, 220 mmol), molecular sieves (10 g), and mercuric cyanide (26 g, 103 mmol) in dry dichloromethane (100 mL). The mixture was stirred for 16 h at room temperature, filtered through a bed of Celite, and the filtrate washed successively with water, M potassium bromide, and water, dried, and evaporated, to give syrupy **3** (27 g, 76%); $[\alpha]_D$ −65° (*c* 1.0, chloroform). Compound **3** (26 g) was dissolved in dry methanol (100 mL), and sodium methoxide (250 mg) was added. The solution was kept for 2 h at room temperature, decationized with Amberlite IR-120 (H^+) resin, the suspension filtered, and the filtrate evaporated to syrupy **4** (15 g, 93%); $[\alpha]_D$ −49° (*c* 1.0, chloroform), lit.[27] −56°.

Allyl 2,3-O-isopropylidene-α-L-rhamnopyranoside (**5**). — Acetone (30 mL) was added to compound **4** (13 g, 64 mmol), followed by 2,2-dimethoxypropane (30 mL) and *p*-toluenesulfonic acid (250 mg). The mixture was stirred for 1 h at room temperature, the acid neutralized with Amberlite IR-400 (OH^-) resin, and the solution evaporated, to yield syrupy **5** (15 g, 83%); $[\alpha]_D$ −35.0° (*c* 1.0, chloroform), lit.[11] $[\alpha]_D$ −36.6°.

4-O-Benzyl-L-rhamnose (**8**). — To a solution of compound **5** (15 g, 73.7 mmol) in tetrahydrofuran (150 mL) was added sodium hydride (3.2 g), and the mixture was stirred for 4 h, and cooled to 0°. Benzyl bromide (20 mL, 168 mmol) was added, and, after 16 h, methanol to decompose the excess of hydride, and the solvent was evaporated. A solution of the residue in chloroform was washed with water, dried, and evaporated to give syrupy **6**. This was applied to a

column of silica gel, and eluted with solvent *A*. Compound **6** was obtained as a syrup (18 g, 87%); $[\alpha]_D$ −58° (*c* 1.0, chloroform).

Compound **6** (15 g) in trifluoroacetic acid (99%, 200 mL) was kept for 1 h at 0°, disappearance of starting material being monitored by t.l.c. in solvent *D*. The acid was removed by codistillation with toluene, and the solution evaporated to a syrup which, without purification, was dissolved in 8:3:1 (v/v) ethanol–benzene–water (430 mL). Tris(triphenylphosphine)rhodium chloride (4 g) and 1,4-diazabicyclo[2.2.2]octane (2 g) were added, and the mixture was heated under reflux overnight and evaporated to dryness. The residue was washed successively with water, M hydrochloric acid, aqueous sodium hydrogencarbonate, and water, dried by evaporation, dissolved in acetone (250 mL) and M hydrochloric acid (30 mL), and the solution heated under reflux for 1 h, cooled, the acid neutralized with sodium hydrogencarbonate, and the solution evaporated. The product was applied to a column of silica gel and developed with solvent *E*. Compound **8** was obtained as crystals (8 g, 70%); m.p. 127–129°, $[\alpha]_D$ −31.3° (*c* 2.02, chloroform).

Anal. Calc. for $C_{13}H_{18}O_5$: C, 61.41; H, 7.08. Found: C, 61.44; H, 7.00.

3-O-Acetyl-4-O-benzyl-1,2-O-(1-methoxyethylidene)-β-L-rhamnopyranose (**11**). — Acetic anhydride (10 mL) was added to an ice-cooled solution of **8** (5 g) in pyridine (15 mL). After 16 h at room temperature, t.l.c. showed the formation of a new compound, R_F 0.66 in solvent *B*. Aqueous processing gave 1,2,3-tri-*O*-acetyl-4-*O*-benzyl-L-rhamnopyranose (**9**) as a syrup (7 g, 93%); $[\alpha]_D$ −35° (*c* 0.5, chloroform), which was used without purification.

Compound **9** (2.5 g, 6.5 mmol) in anhydrous dichloromethane (40 mL) was cooled to −10°, trimethylsilyl bromide (10 mL) was added dropwise, and the mixture was stirred for 3 h at room temperature. T.l.c. showed a faster-moving component having R_F 0.43 in solvent *J*, along with traces of starting material. The solvent and the excess of reagent were evaporated, and 2,6-lutidine (8 mL) and anhydrous methanol (1.2 mL) were added to syrupy bromide **10** (which contained at least 80% of the bromide, as judged by n.m.r. spectroscopy). A white precipitate appeared after 3 h, and the reaction was continued with stirring for 48 h; dichloromethane (10 mL) was then added, and stirring was continued for another 16 h. Filtration, dilution of the filtrate with ethyl acetate (5 mL), extraction with cold water, drying, and evaporation yielded a residue which crystallized on storage (1.4 g, 65%), R_F 0.76 in solvent *b*; m.p. 90°, $[\alpha]_D$ +25° (*c* 1.0, chloroform).

Anal. Calc. for $C_{18}H_{24}O_7$: C, 61.30; H, 6.81. Found: C, 61.50; H, 6.79.

4-O-Benzyl-1,2-O-(1-methoxyethylidene)-3-O-methyl-β-L-rhamnopyranose (**13**). — A solution of compound **11** (1.5 g) in dry methanol was treated with ammonia-saturated methanol (8 mL) and kept for 72 h at room temperature. T.l.c. then showed the disappearance of the starting material and formation of a new, slower-moving component with R_F 0.28 in solvent *B*. The syrupy product **12** obtained on evaporation was used directly for the next step. A solution of the compound in anhydrous tetrahydrofuran was cooled to 0°, and a suspension of sodium hydride in tetrahydrofuran was added. The mixture was stirred for 2 h,

cooled to 0°, methyl iodide added, and stirring continued overnight at room temperature. Processing was conducted by addition of methanol followed by extraction with water and dichloromethane. The organic phase was washed with water, dried, and evaporated, and the syrupy compound was purified by column chromatography using alumina (basic) and solvent *B*. Compound **13** was obtained as a syrup (1.0 g, 72%); $[\alpha]_D$ +29.8° (*c* 6.32, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.26 (d, 1 H, $J_{1,2}$ 2.3 Hz, H-1), 4.8–4.5 (q, 2 H, *J* 10.8 Hz, CH_2Ph), 4.49 (dd, 1 H, $J_{2,3}$ 4.0, $J_{1,2}$ 2.3 Hz, H-2), 3.5 (s, 3 H, OMe), 3.2 (s, 3 H, OMe), 1.64 (s, 3 H, CMe), and 1.24 (d, 3 H, H-6).

Anal. Calc. for $C_{17}H_{24}O_6$: C, 62.96; H, 7.40. Found: C, 62.50; H, 7.10.

*8-(Methoxycarbonyl)octyl 2-O-acetyl-4-O-benzyl-3-O-methyl-α-*L*-rhamnopyranoside* (**14**). — Orthoester **13** (1.1 g, 3.6 mmol) was heated for 2 h under reflux with 8-(methoxycarbonyl)octanol (700 mg, 3.7 mmol) in acetonitrile (12 mL) containing mercuric bromide (50 mg). The solvent was distilled off, and dichloromethane (5 mL) was added. Washing with cold water, drying, evaporation, and chromatography on a column of silica gel with solvent *C* gave pure **14** (1 g, 62%); $[\alpha]_D$ −21.3° (*c* 0.54, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.27 (dd, 1 H, $J_{1,2}$ 1.39, $J_{2,3}$ 3.5 Hz, H-2), 4.63–4.6 (q, 2 H, *J* 10.8 Hz, CH_2Ph), 4.69 (d, 1 H, $J_{1,2}$ 1.39 Hz, H-1), 3.66 (s, 3 H, COOMe), 3.8–3.2 (m, 3 H, ring C*H*), 3.44 (s, 3 H, OMe), 2.3 (t, 2 H, CH_2CO–), 2.15 (s, 3 H, CH_3CO), and 1.7–1.2 [m, 15 H, 3 H-6 and –$(CH_2)_6$–].

Anal. Calc. for $C_{26}H_{40}O_7$: C, 67.24; H, 8.62. Found: C, 66.80; H, 8.33.

*8-(Methoxycarbonyl)octyl 4-O-benzyl-3-O-methyl-α-*L*-rhamnopyranoside* (**15**). — Compound **14** was *O*-deacylated in methanol (20 mL) containing sodium methoxide (100 mg). Decationization with Dowex (H^+) resin, and evaporation of the solvent gave pure **15** in almost quantitative yield; $[\alpha]_D$ −62° (*c* 0.86, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 7.4 (m, 5 H, aromatic), 4.8–4.5 (q, 2 H, *J* 10.9 Hz, CH_2Ph), 4.8 (d, 1 H, $J_{1,2}$ 1.2 Hz, H-1), 3.7 (s, 3 H, COOMe), 3.3 (s, 3 H, OMe), 2.4 (bs, 1 H, O*H*), and 2.3 (d, 2 H, CH_2CO); ^{13}C-n.m.r. ($CDCl_3$): δ 99.2 (C-1), 81.9 (OCH_2Ph), 80.2 (C-3), 75.1 (C-4), 68.2 (C-2), 67.6 (OCH_2), 67.2 (C-5), 57.3 (OMe), 51.2 (COOMe), and 17.9 (C-6).

Anal. Calc. for $C_{24}H_{38}O_7$: C, 65.70; H, 8.67. Found: C, 65.35; H, 8.60.

*8-(Methoxycarbonyl)octyl 4-O-benzyl-2,3-di-O-methyl-α-*L*-rhamnopyranoside* (**16**). — To the partially protected compound **15** (0.5 g, 1.14 mmol) in dichloromethane (2.5 mL) was added 2,6-di-*tert*-butylpyridine (1 mL) followed by methyl trifluoromethanesulfonate (1.2 mL). The solution was heated for 2.5 h at 80°, and cooled to room temperature. T.l.c. then showed the disappearance of the starting material and emergence of a faster-moving component, R_F 0.58 in solvent *G*. Evaporation of the solvent followed by chromatography on a column of silica gel gave compound **16** (0.420 g, 80%); $[\alpha]_D$ −80° (*c* 0.87, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 7.4 (m, 5 H, aromatic), 4.9–4.5 (q, 2 H, *J* 10.8 Hz, CH_2Ph), 4.82 (d, 1 H, $J_{1,2}$ 1.0 Hz, H-1), 3.66 (s, 3 H, COOMe), 3.52 (2 s, 6 H, 2 MeO), 3.7–2.6 (m, ring C*H*), 2.3 (t, 2 H, CH_2CO–), and 1.6–1.2 [m, 15 H, 3 H-6 and –$(CH_2)_6$–];

^{13}C-n.m.r. ($CDCl_3$): δ 173.8 (*C*=O), 97.2 (C-1), 81.9 (O*C*H_2Ph), 80.7 (C-3), 76.5 (C-2), 75.1 (C-4), 67.8 (O*C*H_2), 67.6 (C-5), 58.8, 57.5, 51.1 (3 MeO), and 17.9 (C-6).

To compound **15** (20 mg, 0.05 mmol) in dichloromethane cooled to −10° was added a catalytic amount of boron trifluoride etherate, followed by diazomethane until the faint-yellow color persisted. The procedure was repeated several times, and aqueous processing followed by evaporation of the solvent gave a syrupy product which, when chromatographed through a column of silica gel with solvent *H*, gave compound **16** (12.5 mg, 65%). This compound had physical constants identical to those of **16** prepared by use of methyl trifluoromethanesulfonate.

Anal. Calc. for $C_{25}H_{40}O_7$: C, 66.37; H, 8.84. Found: C, 66.00; H, 8.45.

*8-(Methoxycarbonyl)octyl 2,3-di-*O*-methyl-α-*L*-rhamnopyranoside* (**17**). — Hydrogenolysis of compound **16** (20 mg) in methanol (2 mL) for 24 h at room temperature under atmospheric pressure, using palladium-on-charcoal (10%; 20 mg), gave **17** as a syrup; R_F 0.2 in solvent *I*; $[\alpha]_D$ −47° (*c* 0.93, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 4.72 (d, 1 H, $J_{1,2}$ 1.6 Hz, H-1), 3.52 (s, 3 H, COOMe), 3.36, 3.33 (2 s, 6 H, 2 MeO), 2.20 (t, 3 H, C*H*$_2$CO), and 1.6–1.1 (m, 15 H); ^{13}C-n.m.r. ($CDCl_3$): δ 97.3 (C-1), 81.3 (C-3), 76.5 (C-2), 71.7 (C-4), 68.3 (O*C*H_2), 67.4 (C-5), 58.6, 56.8, 50.9 (3 MeO), and 17.5 (C-6).

Anal. Calc. for $C_{18}H_{34}O_7$: C, 59.66; H, 9.39. Found: C, 59.25; H, 9.13.

8-(Methoxycarbonyl)octyl O-*(2,4-di-*O*-acetyl-3,6-di-*O*-methyl-β-*D*-glucopyranosyl)-(1→4)-2,3-di-*O*-methyl-α-*L*-rhamnopyranoside* (**19**). — To a stirred solution of compound **17** (0.100 g, 0.27 mmol) in dichloromethane containing 5:2 mercuric cyanide–mercuric bromide (0.35 mmol) and powdered molecular sieves was added a solution of 2,4-di-*O*-acetyl-3,6-di-*O*-methyl-D-glucopyranosyl bromide[14] (0.175 g, 0.49 mmol) in dichloromethane, and the mixture was stirred for 16 h at room temperature in an inert atmosphere. T.l.c. (solvent *F*) then showed the formation of a new component, R_F 0.43, accompanied by some hydrolyzed by-product. The solids were removed by filtration, and washed with dichloromethane, and the filtrate and washings were combined, washed successively with water, M potassium bromide, and water, dried, and evaporated. Compound **19** was applied to a small column of silica gel, using solvent *F*, resulting in 70% recovery (0.125 g); $[\alpha]_D^{20}$ −12.4° (*c* 0.221, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.3–4.5 (m, 4 H, H-1,1′,2′,4′), 4–3.3 (m, 5 MeO and ring C*H*), 2.44 (t, 2 H, C*H*$_2$CO–), 2.15 (s, 3 H, C*H*$_3$CO–), 2.05 (s, 3 H, C*H*$_3$CO–), and 1.8–1.0 [m, 15 H, 3 H-6 and –(C*H*$_2$)$_6$–]; ^{13}C-n.m.r. ($CDCl_3$): δ 100.9 (C-1′), 96.8 (C-1), 81.5 (C-3), 77.7 (C-4), 76.8 (C-2), 73.0 (C-6′), 72.6 (C-5′), 72.2 (C-2′), 70.4 (C-4′), 67.7 (O*C*H_2), 67.1 (C-5), 59.4, 58.7, 58.0, 56.9, 51.1 (5 MeO), and 17.6 (C-6).

8-(Methoxycarbonyl)octyl O-*(3,6-di-*O*-methyl-β-*D*-glucopyranosyl)-(1→4)-2,3-di-*O*-methyl-α-*L*-rhamnopyranoside* (**20**). — Compound **19** in anhydrous methanol was cooled to 0°, and dry sodium methoxide (15 mg) added. The solution was kept overnight at room temperature, and after 16 h, was de-ionized and evaporated, to afford a homogeneous syrup; $[\alpha]_D$ −26.8° (*c* 0.335, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 4.9 (s, 1 H, H-1), 4.5 (d, 1 H, $J_{1',2'}$ 7.81 Hz, H-1′), 3.95 (s, 1

H, H-2), 3.67, 3.66, 3.49, 3.47, 3.39 (5 s, 15 H, 5 MeO), 2.8 (d, 1 H, O*H*), and 2.3 (t, 2 H, CH_2CO); ^{13}C-n.m.r. (CD_3OD): δ 105.0 (C-1'), 98.5 (C-1), 87.6 (C-3'), 82.4 (C-3), 80.7 (C-4), 78.0 (C-2'), 76.7 (C-2), 75.5 (C-5'), 73.2 (C-6'), 71.3 (C-4'), 68.8 (–O*C*H$_2$), 68.7 (C-5), 60.5–51.8 MeO), and 18.1 (C-6).

Anal. Calc. for $C_{26}H_{48}O_{12}$: C, 56.52; H, 8.69. Found: C, 56.30; H, 8.60.

8-Carbazoyloctyl O-(3,6-di-O-methyl-β-D-glucopyranosyl)-(1→4)-2,3-di-O-methyl-α-L-rhamnopyranoside (**21**). — The partially deblocked disaccharide **20** (0.090 g) in ethanol (1.5 mL) was treated with hydrazine hydrate (0.5 mL) for 24 h at room temperature. After evaporation, and codistillation of traces of hydrazine with ethanol, the residue was dried under vacuum. Purification on a column of LH-20 (solvent *D*) gave pure **21** (0.080 g, 85%); $[\alpha]_D$ −5° (*c* 0.92, H_2O); ^{1}H-n.m.r. (CD_3OD): δ 4.8 (bs, 1 H, H-1), 4.5 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 3.8–2.8 (m, ring C*H* and 4 MeO), 2.20 (t, 2 H, CH_2CO–), and 1.7–1.1 [m, 15 H, 3 H-6 and –(CH_2)$_6$–]; ^{13}C-n.m.r. (CD_3OD): δ 105.0 (C-1'), 98.4 (C-1), 87.6 (C-3'), 82.5 (C-3), 79.7 (C-4), 77.9 (C-2'), 76.8 (C-2), 75.5 (C-5'), 73.1 (C-6'), 71.3 (C-4'), 68.8 (–O*C*H$_2$), 68.6 (C-5), 60.8, 59.7, 59.1 (4 MeO), and 18.6 (C-6).

Anal. Calc. for $C_{25}H_{48}N_2O_{11}$: C, 54.34; H, 8.69; N, 5.07. Found: C, 54.30; H, 8.71; N, 5.00.

O-(3,6-Di-O-methyl-β-D-glucopyranosyl)-(1→4)-O-(2,3-di-O-methyl-α-L-rhamnopyranosyl)-(1→9)-oxynonanoyl-BSA ("natural disaccharide-octyl-BSA"; ND-O-BSA) (**22**). — A stirred solution of the hydrazide (11 μmol) in dry *N,N*-dimethylformamide (125 μL) was cooled to −30°, and 3.6M HCl–1,4-dioxane (25 μL) was added. *tert*-Butyl nitrite in *N,N*-dimethylformamide (1:10; 40 μL) was added, and the solution was stirred for 30 min at −30°. T.l.c. in solvent *K* then showed the disappearance of the hydrazide and formation of a new, faster-moving component. The excess of nitrous acid was neutralized with 0.5M sulfamic acid (40 μL). The cold (−50°) solution of acyl-azide was added dropwise, by means of a micropipet precooled to −70°, to a solution of BSA (8 mg, 0.1 μmol) in 0.08M $Na_2B_4O_7$ (0.8 mL) and 0.3M $KHCO_3$, pH 9.2, at 0°, stirred overnight at 0°, and dialyzed against four changes of de-ionized water in an ultrafiltration cell (Amicon Corp., Danvers, MA) equipped with a PM-10 membrane. Gel filtration of the dialysis retentate was conducted in a column (80 × 1.6 cm) of Sephadex G-75 in phosphate-buffered saline. The neoglycoprotein solution was lyophilized in 0.5–1.0-mL aliquots, and stored at −10°.

Other neoglycoproteins. — Details of the synthesis of *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranose have been described[12], as has its coupling to BSA by reductive amination, to produce the neoglycoprotein *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-(1-deoxy-2,3-di-*O*-methyl-L-rhamnitol-1-yl)-BSA (reduced disaccharide-BSA; rD-BSA)[13].

Likewise, the synthesis of *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→9)-oxynonanoyl-BSA (monosaccharide-octyl-BSA; M-O-BSA) and of *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→9)-oxynonanoyl-BSA (disaccharide-octyl-BSA; D-O-BSA) has been described[14]

Serology. — The only modification of the ELISA protocol developed originally[5] for the native PGL-I and the rD-BSA was the inclusion of 0.1% Tween in the blocking buffer. IgM only was assayed, as the majority of anti-PGL-I antibodies in leprosy sera are of this immunoglubulin class[5,28].

Sera were obtained (from Robert H. Gelber, M.D.) from individual leprosy patients attending the Hansen's Disease Clinic, Seton Medical Center, Daly City, CA, or Thomas H. Rea, M.D., Los Angeles County-University of Southern California, Los Angeles, CA. Patients were classified clinically and pathologically according to the Ridley and Jopling scale[29]. Also, three reactional states, namely, reversal reactions, erythema nodosum leprosum (ENL), and Lucio reaction, were diagnosed according to published criteria[30]. Sera were also obtained from patients with mycobacterial infections, other than *M. leprae,* attending the National Jewish Center for Immunology and Respiratory Medicine, Denver, CO. Normal sera were obtained from individuals in the indigenous population of Colorado.

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