Carbohydrate Research, 183 (1988) 241–260
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

SYNTHESIS AND IMMUNOREACTIVITY OF NEOGLYCOPROTEINS CONTAINING THE TRISACCHARIDE UNIT OF PHENOLIC GLYCOLIPID I OF *Mycobacterium leprae*

DELPHI CHATTERJEE, SANG-NAE CHO*, CAROL STEWART, JAMES T. DOUGLAS†, TSUYOSHI FUJIWARA‡, AND PATRICK J. BRENNAN**

Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523 (U.S.A.)

(Received October 20th, 1987; accepted for publication in revised form, May 9th, 1988)

ABSTRACT

The trisaccharide segment, *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-L-rhamnopyranose, of the *Mycobacterium leprae*-specific phenolic glycolipid I has been synthesized as its 8-(methoxycarbonyl)octyl glycoside and coupled to a carrier protein, to produce a leprosy-specific neoglycoprotein, the so-called natural trisaccharide-octyl-bovine serum albumin (NT-O-BSA). Special features of the synthetic strategy were the use of silver trifluoromethanesulfonate (triflate) to promote glycosylation, resulting in the rhamnobiose in high yield and absolute stereospecificity. The terminal 3,6-di-*O*-methyl-D-glucopyranosyl group was introduced after *O*-deallylation of the rhamnobiose. Removal of protecting groups yielded the trisaccharide hapten suitable for coupling to carrier protein. Poly(acrylamide)-gel electrophoresis of the neoglycoprotein demonstrated its purity, and subsequent immunoblotting with a monoclonal antibody directed to the terminal 3,6-di-*O*-methyl-β-D-glucopyranosyl epitope of the native glycolipid demonstrated its antigenicity. Comparative serological testing in enzyme-linked immunosorbent assays of NT-O-BSA, the corresponding disaccharide-containing products, and another trisaccharide-containing neoglycoprotein, *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-rhamnopyranosyl)-(1→4′)-oxy-(3-phenylpropanoyl)-BSA (NT-P-BSA) [Fujiwara *et al.*, *Agric. Biol. Chem.*, 51 (1987) 2539–2547] against sera from leprosy patients and control populations showed concordance; the presence of the innermost sugar did not contribute significantly to sensitivity or specificity. The di- and tri-saccharide-containing neoantigens, on account of ready availability and solubility, provide greater flexibility than the native glycolipid for the serodiagnosis of leprosy.

*Present address: Department of Microbiology, Yonsei University, College of Medicine, Seoul 120-749, Korea.
†Present address: Department of Microbiology, University of Hawaii, Honolulu, Hawaii 96822, U.S.A.
‡Present address: Institute for Natural Science, Nara University, Horai-cho 1230, Nara, Japan.
**To whom correspondence should be addressed.

0008-6215/88/$ 03.50

INTRODUCTION

A variety of neoglycoproteins containing the terminal glycobiose unit, *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranosyl, of the *Mycobacterium leprae*-specific glycolipid antigen, phenolic glycolipid-I (PGL-I)[1], have been synthesized in this laboratory, and have proved highly active and specific when allowed to react against lepromatous leprosy sera in enzyme-linked, immunosorbent assays (ELISA)[2–5]. The most significant of these is *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-α-L-rhamnopyranosyl)-(1→9)-oxynonanoyl-BSA (ND-O-BSA). Compared to the native glycolipid, the neoglycoproteins are advantageous because of their water-solubility, which makes them suitable for use in such procedures as dot-nitrocellulose-ELISA[6] and latex agglutination tests[7], and suitable for field applications.

Fujiwara and associates[8,9] have also independently synthesized a series of neoglycoproteins containing the proper nonreducing-end disaccharide epitope and modifications thereof, but having a phenylpropanoyl segment linking the sugar to BSA (the most important of these is the so-called natural disaccharide-phenylpropanoyl-BSA, ND-P-BSA)[8,9]. Likewise, Brett *et al.*[10], by using the terminal synthetic disaccharide and its propyl glycoside, prepared by Gigg *et al.*[11], generated and tested several relevant glycoconjugates. In extensive comparative ELISA of a wide range of serum specimens from patients with lepromatous and tuberculoid leprosy and tuberculosis, and from normal control populations, satisfactory concordance among all of the available semi-synthetic antigens was observed[5,12]. Clearly, then, the available disaccharide-based neoglycoproteins are highly suitable for the serodiagnosis of lepromatous leprosy; indeed, they are in use world-wide for this purpose[13].

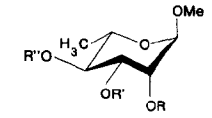
However, our earlier study also showed that, among a large collection of lepromatous leprosy sera, there were some that were reactive to the complete native antigen, PGL-I, but that did not respond to ND-O-BSA[5], implying that such antibodies preferentially recognize the reducing-end, 3-*O*-methyl-L-rhamnopyranosyl unit of PGL-I. Accordingly, we undertook synthesis of the entire trisaccharide hapten, a feat that had been previously accomplished[2,14,15], but this time in the form of a suitable neoglycoprotein, and, by comparing its serological activity to that of the native PGL-I, to the earlier disaccharide-containing neoglycoproteins, and to another trisaccharide-containing neoglycoprotein independently synthesized, arrived at a more complete synthetic tool for the serodiagnosis of leprosy.

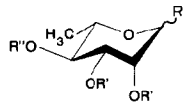
RESULTS AND DISCUSSION

Synthesis of O-*(3,6-di-*O*-methyl-β-*D*-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-α-*L*-rhamnopyranosyl)-(1→2)-*O*-(3-*O*-methyl-α-*L*-rhamnopyranosyl)-(1→9)-oxynonanoyl-BSA (natural trisaccharide-octyl-BSA; NT-O-BSA).* — Our

approach to the synthesis of the trisaccharide unit of phenolic glycolipid I of *M. leprae* was to construct the molecule sequentially, starting from the reducing end. As in our earlier work with disaccharides[4,5], the triglycosyl unit was elaborated as a glycoside of 8-(methoxycarbonyl)octanol[16]. To establish the (1→2)-linkage, the method developed by Borén *et al.*[17], as later applied by Bundle and Josephson[18], was exploited. The selectively blocked **11** (having a free 2-hydroxyl group) was used as both the glycosyl acceptor and a modified, reducing-end sugar. In order to generate the internal rhamnosyl residue of the trisaccharide unit in the α configuration an orthoacetate having a temporary protecting group at OH-4 was used; this was then converted into glycosyl donor **10**. Upon silver triflate-promoted glycosylation, the disaccharide **12** was obtained.

1,2,3-Tri-*O*-acetyl-4-*O*-allyl-α,β-L-rhamnopyranose (**5**) was synthesized from the known methyl 4-*O*-allyl-2,3-*O*-isopropylidene-α-L-rhamnopyranoside[19] (**2**) by acid hydrolysis to the free sugar **4**, followed by conversion into its triacetate (**5**). Compound **5** was converted into the corresponding bromide (**6**) by using hydrogen



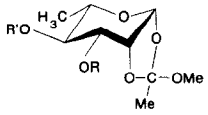


	R	R′	R″
1	CMe_2		H
2	CMe_2		All
3	H	H	All

	R	R′	R″
4	OH	H	All
5	OAc	Ac	All
6	Br	Ac	All

All = $H_2C{=}CHCH_2$

bromide in glacial acetic acid, and treatment of **6** with anhydrous methanol in the presence of 2,6-lutidine gave 3-*O*-acetyl-4-*O*-allyl-1,2-*O*-(1-methoxyethylidene)-β-L-rhamnopyranose (**7**). This syrupy product was deacetylated, and the product (**8**) was methylated[20] at O-3, to give **9**. Pure homogeneous compounds, according to t.l.c. and n.m.r.-spectral analysis, were obtained at all steps, so that no chromatographic purifications were required. Traces of 2,6-lutidine remaining in the isolated products were not removed, except for analytical purposes, when small amounts of samples were purified by column chromatography on basic alumina.



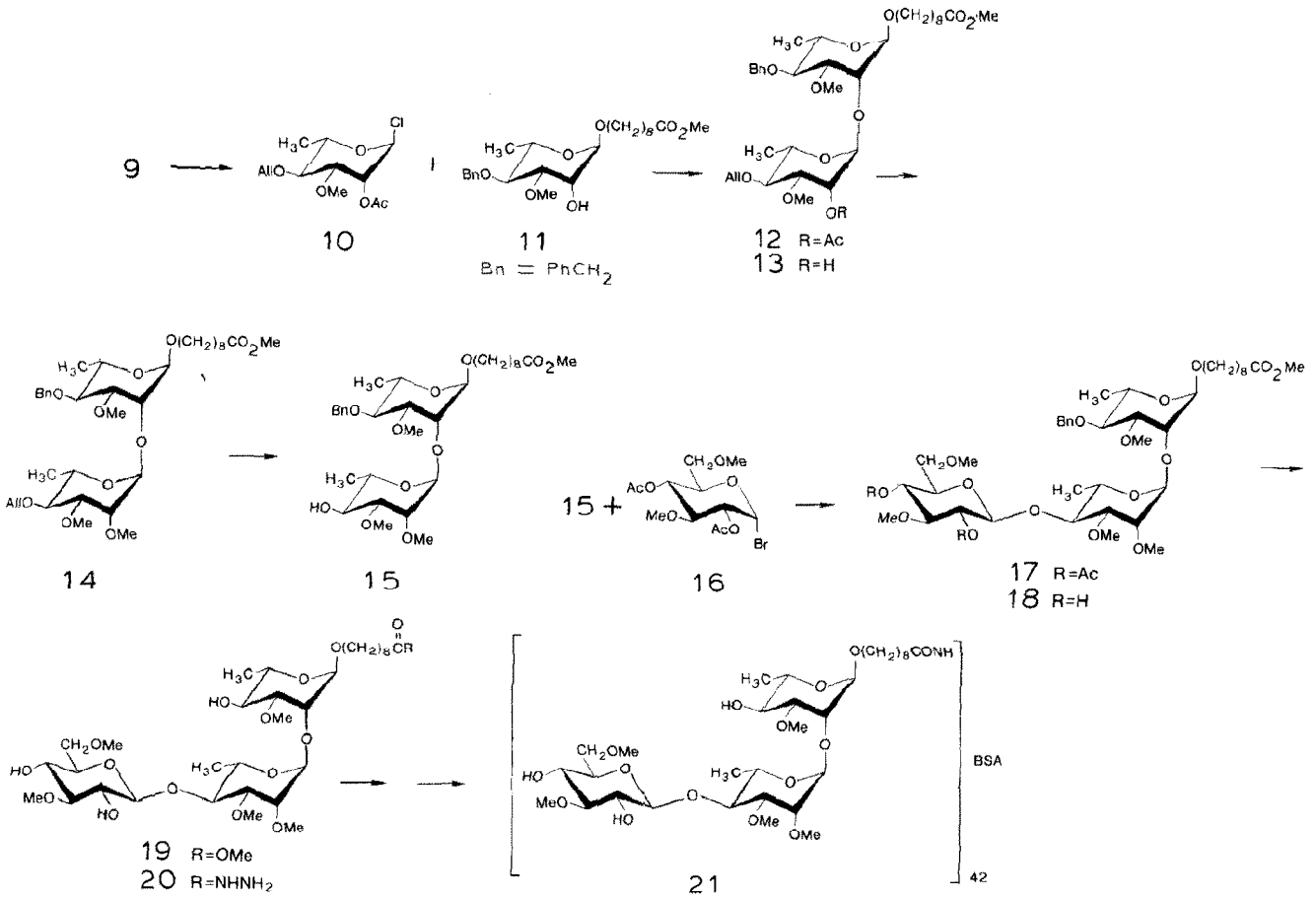
	R	R′
7	Ac	All
8	H	All
9	Me	All

The orthoester **9** was quantitatively converted into 2-*O*-acetyl-4-*O*-allyl-3-*O*-methyl-α-L-rhamnopyranosyl chloride (**10**) by using chlorotrimethylsilane[21]; efforts to obtain the corresponding bromide through use of bromotrimethylsilane were thwarted by concomitant *O*-demethylation. The silver-assisted Koenigs-Knorr condensation[22] of **10** with **11** (ref. 5) produced the disaccharide **12** in 70% yield after column chromatography. The disaccharide **12** was then *O*-deacetylated to **13**. The anomeric purity of the disaccharide **13** was confirmed by ^{13}C- and ^{1}H-n.m.r. spectroscopy. The ^{1}H-n.m.r. spectrum showed well separated, narrow doublets at δ 5.2 and 4.7 for H-1′ and H-1, perhaps indicating the α configuration, and this was substantiated by the ^{13}C shifts of the signals of C-1′ and C-1 at δ 103.0 ($^{1}J_{CH}$ 169 Hz) and 100.5 ($^{1}J_{CH}$ 168.7 Hz).

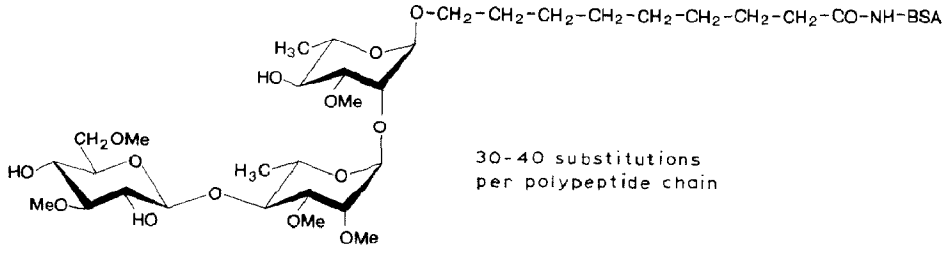
Compound **13** could be methylated with methyl triflate to give **14**, but when the reaction was conducted on a large scale, a considerable amount of degradation was noticed. The methylation of **13** was therefore accomplished by following Brimacombe's protocol[20], and it proceeded satisfactorily when an equimolar quantity of sodium hydride was used. In this way, the fully substituted intermediate **14** was obtained in quantitative yield. Use of Wilkinson's catalyst in the presence of 1,4-diazabicyclo[2.2.2]octane isomerized the allyl group to a 1-propenyl group, and cleavage of the propenyl ether with mercuric chloride and mercuric oxide for 30 min at room temperature gave the disaccharide acceptor **15** in 85% yield. Coupling of **15** with 2,4-di-*O*-acetyl-3,6-di-*O*-methyl-β-D-glucopyranosyl bromide (**16**, freshly prepared[4] from its precursor triacetate) in the presence of 5:2 mercuric cyanide–mercuric bromide in anhydrous dichloromethane gave the fully protected trisaccharide **17**.

The anomeric configuration of the newly introduced glycosidic linkage was confirmed by n.m.r. spectroscopy after *O*-deacetylation of **17** to **18**. The ^{1}H-n.m.r. spectrum of compound **18** showed a doublet for H-1″ at δ 4.4 ($J_{1'',2''}$ 7.5 Hz), and, in the ^{13}C-n.m.r. spectrum, the signal for C-1″ was at δ 105.0 ($^{1}J_{CH}$ 161.5 Hz), consistent with a β-D-hexosyl linkage. *O*-Debenzylation of **18** gave **19**. To confirm its sugar composition, the trisaccharide **19** was hydrolyzed with 2M trifluoroacetic acid at 121°, and the products were reduced, the alditols acetylated, and the acetates examined by g.l.c.–m.s. on 3% of SP-2340. Only the three partially methylated, partially acetylated alditols expected were found: 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylrhamnitol (*m/z* 203, 161, 143, 117, and 101); 1,2,4,5-tetra-*O*-acetyl-3-*O*-methylrhamnitol (*m/z* 203, 189, 143, and 129); and 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylglucitol (*m/z* 233, 189, 129, 113, and 87).

Compound **19** was quantitatively converted into its hydrazide (**20**). This was purified by chromatography on a column of LH 20, freeze-dried, and then converted into the acyl azide, which was immediately conjugated to the carrier protein, namely, bovine serum albumin (BSA)[4,5]. The resulting neoglycoconjugate was dialyzed, and subjected to gel filtration on Sephadex G-75. The carbohydrate in the effluent corresponding to the void volume was analyzed for protein absorption at 280 nm, and for hapten content by carbohydrate assay as described[5]. An

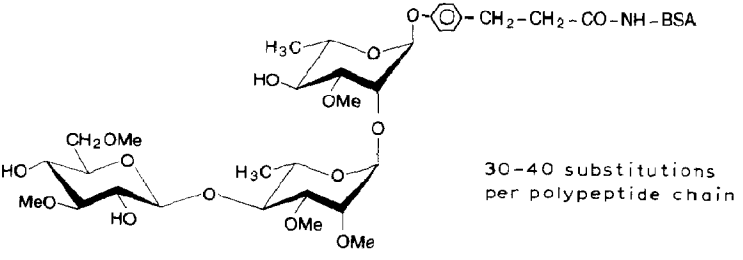

9
10
11
Bn = PhCH2
12 R=Ac
13 R=H
14
15
15 +
16
17 R=Ac
18 R=H
19 R=OMe
20 R=NHNH2
21
BSA
42

incorporation ratio of 40–45 mol of hapten per mol of BSA was achieved. The structure of the resulting *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-α-L-rhamnopyranosyl)-(1→2)-*O*-(3-*O*-methyl-α-L-rhamnopyranosyl)-(1→9)-oxynonanoyl-BSA, designated natural trisaccharide-octyl-BSA (NT-O-BSA, **21**) is presented, along with that of the 3-phenylpropanoate-linked neoglycoprotein[9], *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-α-L-rhamnopyranosyl)-(1→2)-*O*-(3-*O*-methyl-α-L-rhamnopyranosyl)-(1→4′)-oxy-(3-phenylpropanoyl)-BSA (**22**). Neoglycoprotein **22** also carries ~40 haptenic groups per molecule.



21

Natural trisaccharide-octyl-BSA (NT-O-BSA)



22

Natural trisaccharide-phenylpropanoyl-BSA (NT-P-BSA)

P.a.g.e. and Western blotting of the neoglycoproteins. — Poly(acrylamide) gel electrophoresis (p.a.g.e.) proved to be an effective means of assessing the purity, homogeneity, and relative molecular weight (M_r) of the neoglycoproteins. P.a.g.e. of the octyl-linked disaccharide-containing neoglycoproteins, *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)[4], and *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)[5], along with NT-O-BSA and NT-P-BSA, showed an impressive degree of purity (see Fig. 1A); the NT-P-BSA preparation is not shown. Based on the molecular mass of underivatized BSA

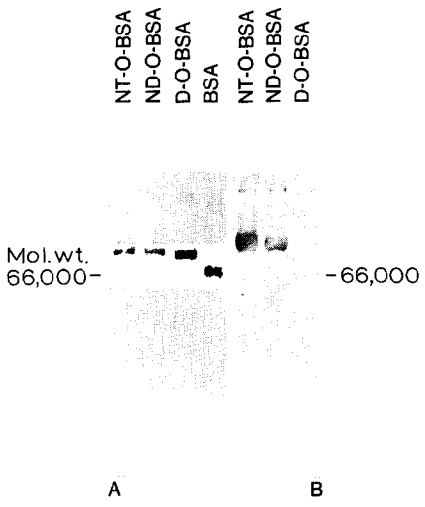


Fig. 1. Poly(acrylamide) gel electrophoresis (p.a.g.e.) of three of the leprosy-specific neoglycoproteins. A. Gel stained with Coomassie Blue. B. Results of electrophoretic transfer of the neoglycoproteins from poly(acrylamide) gel to nitrocellulose, and immunoblotting with monoclonal antibody 33.13.

(66,000), it was possible to gauge the M_r values of the neoglycoproteins as being in the region of 90,000–100,000, which is to be expected from BSA substituted with 40-47 molecules of hapten per molecule of protein. Western-style immunoblotting, using a monoclonal antibody raised against PGL-I and directed to the 3,6-di-*O*-methyl-β-D-glucopyranosyl epitope, was also applied to some of the available neoglycoproteins, in order to assess both purity and immunoreactivity (see Fig. 1B). The NT-O-BSA, ND-O-BSA, and NT-P-BSA (not shown) reacted; however, the D-O-BSA did not bind to the antibody. The results clearly demonstrated the strong immunoreactivity of the neoglycoconjugates containing the native di- or tri-glycosyl unit, and emphasized the role of the *O*-methyl groups of the penultimate L-rhamnosyl residue in antibody binding, at least to this monoclonal antibody. The importance of the *O*-methyl groups of the terminal D-glucopyranosyl group of PGL-I in recognizing human polyclonal, anti-glycolipid IgM (ref. 2) and other IgM (ref. 23) and IgG (refs. 2 and 25) monoclonal antibodies had already been demonstrated, and the penultimate di-*O*-methyl-L-rhamnopyranosyl residue seemed not to play a significant role in the antigenicity of PGL-I (refs. 24 and 25). Accordingly, the acute need for *O*-methyl groups on the penultimate sugar appears to be a singular property of antibody 33.13. The activity of variable quantities of NT-O-BSA against pooled lepromatous leprosy serum (results not shown) demonstrated that a highly active glycoconjugate, applicable to the serodiagnosis of lepromatous leprosy, has now been synthesized.

Relative efficacy of the PGL-based neoglycoproteins in the serodiagnosis of leprosy. — Comparative studies were conducted with PGL-I, ND-O-BSA,

NT-O-BSA, and NT-P-BSA, in order to assess the relative sensitivity and specificity of these antigens in detecting anti-glycolipid antibodies in leprosy sera. A total of 199 serum specimens was obtained from leprosy patients presenting themselves to R. H. Gelber, M.D., at the Clinic of the U.S. Public Health Service, Seton Medical Center, Daly City, CA. Patients had been classified, clinically and pathologically, according to the Ridley and Jopling[26] scale. The duration of chemotherapy, applied as described by Gelber[27], was recorded. The origin of the healthy control and tuberculosis populations has been described[3,28]. Indirect ELISA[29] with modification[28] was employed.

A comparison is made in Table I of the activity of PGL-I and NT-O-BSA against sets of sera from lepromatous and tuberculoid patients who had been subjected to chemotherapy for the indicated periods. In general, there was good agreement between the two antigens in ability to detect anti-PGL-I IgM antibodies. Of 199 tested, 97 (48.8%) were seropositive to PGL-I, and 106 (52.3%) to NT-O-BSA. NT-O-BSA thus gave higher seropositivity than PGL-I, although the difference between the two antigens was not statistically significant (McNemar's test, $0.10 > p > 0.05$). Also, sera from tuberculoid and long-time treated lepromatous patients with low level antibody to PGL-I reacted more readily to NT-O-BSA; however, with a specificity rate of 98.2 and 95.9%, respectively, there was no significant difference between the two antigens (McNemar's test, $p > 0.10$). More importantly, NT-O-BSA showed a lesser propensity to react nonspecifically

TABLE I

COMPARISON OF PGL-I AND NT-O-BSA IN ASSAY OF ANTI-PGL-I IgM ANTIBODIES IN SERA FROM LEPROSY PATIENTS

Patients			*Antigen used*	
Classification[a]	*Time on chemotherapy (yr)*	*Number tested*	*PGL-I positives*[b]	*NT-O-BSA positives*[b]
			No. (%)	*No. (%)*
Tuberculoid (TT + BT)				
	2	29	7 (24.1)	9 (31.0)
	2–4	23	4 (17.4)	6 (26.1)
	5	11	3 (27.3)	3 (27.3)
Lepromatous (BL + LL)				
	2	31	23 (74.2)	23 (74.2)
	2–4	43	31 (72.1)	31 (72.1)
	5–9	34	19 (55.9)	20 (58.8)
	10	28	10 (35.7)	14 (50.0)

[a]Tuberculoid leprosy patients included polar tuberculoid (TT) and borderline tuberculoid (BT), and lepromatous patients included borderline lepromatous (BL) and polar lepromatous (LL). [b]$A_{490} \geq 0.100$.

TABLE II

COMPARISON OF PGL-I AND NT-O-BSA IN ASSAY OF ANTI-PGL-I IgM ANTIBODIES IN SERA FROM HEALTHY CONTROLS AND TUBERCULOSIS PATIENTS

Subjects	*Number tested*	*Antigen used*	
		PGL-I positives[a]	*NT-O-BSA positives*[a]
		No. (%)	*No. (%)*
Healthy controls	116	3 (2.6)	1 (0.9)
Tuberculosis patients	53	4 (9.4)	2 (3.8)
Total	169	7 (4.1)	3 (1.8)

[a] $A_{490} \geq 0.100$.

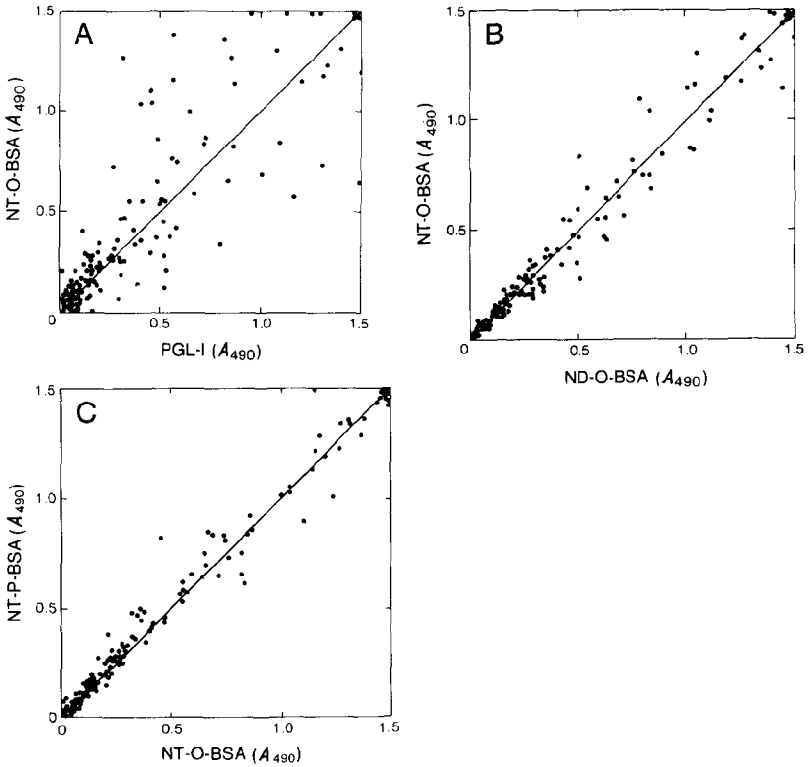


Fig. 2. Pairwise correlations between ELISA values obtained for the individual leprosy sera described in Table I.

TABLE III

COMPARISON OF PGL-I AND THREE NEOANTIGENS IN THE DETECTION OF ANTI-GLYCOLIPID IgM IN SERA FROM LEPROSY PATIENTS

Patients			*Antigen used*			
Classification	*Time on chemotherapy (yr)*	*Number tested*	*PGL-I*	*ND-O-BSA*	*NT-O-BSA*	*NT-P-BSA*
			No. of positives[a]			
Tuberculoid (TT + BT)	2	29	7	9	9	9
	2–4	23	4	6	6	7
	5	11	3	3	3	3
Lepromatous (BL + LL)	2	31	23	23	23	23
	2–4	43	31	31	31	31
	5–9	34	19	20	20	21
	10	28	10	14	14	14
Total		199	97	106	106	108

[a] $A_{490} \geq 0.100$.

TABLE IV

COMPARISON OF PGL-I AND THREE NEOANTIGENS IN THE DETECTION OF ANTI-GLYCOLIPID IgM IN CONTROL GROUPS

Subjects	*Number tested*	*Antigen used*			
		PGL-I	*ND-O-BSA*	*NT-O-BSA*	*NT-P-BSA*
		No. of positives[a]			
Healthy controls	116	3	1	1	1
Tuberculosis patients	53	4	2	2	2
Total	169	7	3	3	3

[a] $A_{490} \geq 0.100$.

with sera from an asymptomatic control population and a group of people with tuberculosis (see Table II). Only three (1.8%) of 169 sera reacted positively to NT-O-BSA, compared to seven (4.1%) for PGL-I. Fig. 2A compares the ELISA absorbances of the individual sera reacting against PGL-I and NT-O-BSA. The absorbance values obtained with the two antigens were well correlated ($r = 0.886$, $p < 0.001$, Student's t-test). The greater reactivity to NT-O-BSA in some sera may reflect greater numbers of antigen determinants.

Three of the major neoglycoproteins in current use for the serodiagnosis of

TABLE V

PAIRWISE COMPARISON OF PGL-I AND SOME NEOANTIGENS IN THE DETECTION OF ANTI-GLYCOLIPID IgM IN SERA FROM LEPROSY PATIENTS[a]

Antigens compared	*Correlation coefficient (r)*	*Percent agreement*[b]
PGL-I *vs.* ND-O-BSA	0.884	91.5
PGL-I *vs.* NT-O-BSA	0.886	91.5
PGL-I *vs.* NT-P-BSA	0.889	90.5
ND-O-BSA *vs.* NT-O-BSA	0.988	100.0
ND-O-BSA *vs.* NT-P-BSA	0.984	99.0
NT-O-BSA *vs.* NT-P-BSA	0.990	99.0

[a]Total number of sera tested was 199. [b]Calculated as: (No. of sera positive to both antigens + No. of sera negative to both antigens)/(total number of sera) × 100.

leprosy, namely, ND-O-BSA, NT-O-BSA, and NT-P-BSA, were compared to PGL-I for ability to detect anti-PGL-I antibodies in the available set of sera (see Table III). The synthetic products, in general, showed greater numbers of seropositive specimens than the native glycolipid, especially when sera from long-term-treated lepromatous patients were being tested. Likewise, the neoantigens showed lesser numbers of seropositive samples among healthy controls and tuberculosis patients (see Table IV). In these respects, ND-O-BSA, NT-O-BSA, and NT-P-BSA yielded virtually identical results.

The correlation coefficients for absorbances between the antigens, and the extent of agreement in establishing seropositivity or seronegativity, were determined (see Table V). The correlation coefficients varied from 0.884 in PGL-I *versus* ND-O-BSA to 0.990 in NT-O-BSA *versus* NT-P-BSA, and all of the antigens were well correlated, with statistical significance ($p < 0.001$, Student's t-test). The agreement in determining seropositivity and seronegativity was ~91% between PGL-I and the synthetic antigens, and >99% between the neoantigens.

The absorbance values of the individual sera against ND-O-BSA and NT-O-BSA were plotted in Fig. 2B, and those against NT-O-BSA and NT-P-BSA in Fig. 2C, respectively. As shown in these Figures, the degree of correspondence between the antigens was nearly perfect, indicating that ND-O-BSA is as efficacious as NT-O-BSA in reacting with anti-glycolipid antibodies; the inner L-rhamnopyranosyl residue does not appear to contribute to immunogenicity or antigenicity. Likewise, the phenyl substituent in NT-P-BSA neither resulted in increased reactivity with leprosy sera nor conferred cross-reactivity against tuberculosis sera (see Table IV).

This extensive study thus demonstrates that the neoantigens ND-O-BSA, NT-O-BSA, and NT-P-BSA are comparable to PGL-I in binding to antiglycolipid antibodies. The expectation that the neoantigens would have greater sensitivity and specificity was not confirmed by the present study. However, the large-scale

utilization of these products, now underway in leprosy-endemic areas, will provide further relevant data. Clearly, the neoantigens, with the advantages of water-solubility and low cost, can now replace the native PGL-I. Clear also was the fact that the presence of the internal sugar substituent conferred no advantage on these neoantigens in the serodiagnosis of leprosy.

EXPERIMENTAL

General methods. — All solvents and reagents were purified and dried according to standard procedures[30]. The anhydrous dichloromethane used as a reaction solvent was purchased from Aldrich Chemical Company (Milwaukee, WI) in "Sure Seal" bottles, and was always transferred and stored under dry argon. For the performance of glycosylations, all glassware was dried overnight in an oven at 120°, and cooled in a desiccator containing P_2O_5 prior to use in reactions. T.l.c. was performed on aluminum plates coated with Silica Gel 60F-254 (E. Merck, Darmstadt, West Germany). The following chromatographic solvent systems were used: *a*, 1:1 chloroform–ether; *b*, 7:1 chloroform–ether; *c*, 9:1 chloroform–ether; *d*, 4:1 chloroform–ether; *e*, 4:1 benzene–ethyl acetate; *f*, 4:1 benzene–acetone; *g*, 9:1 chloroform–methanol; *h*, 7.5:1 chloroform–methanol; *i*, 1:1 ether–ethyl acetate; and *j*, 5:2:0.1 ethyl acetate–methanol–water. Column chromatography was performed on silica gel 60 (60–200 mesh; J. T. Baker Chemical Co., Phillipsburg, NJ), usually 50–70 g per g of sample. For large-scale preparations requiring medium resolution, flash chromatography[31] was routinely employed. Optical rotations were measured with a Perkin–Elmer 241 Polarimeter. N.m.r. spectra were recorded for solutions in chloroform-*d* or methanol-*d*, with tetramethylsilane as the internal standard, using Bruker WH-200 and Bruker WH-270 instruments. Neoglycoconjugates were purified by dialysis followed by gel filtration in phosphate-buffered saline, as described[4,5].

1,2,3-Tri-O-acetyl-4-O-allyl-L-rhamnopyranose (**5**). — A solution of methyl 4-*O*-allyl-2,3-*O*-isopropylidene-α-L-rhamnopyranoside (**2**; 20 g) in dichloromethane (150 mL) was cooled to 0°. Trifluoroacetic acid (99%; 100 mL) was added, and the mixture was kept for 30 min at 0°; t.l.c. in solvent *b* then showed the disappearance of the starting material. The acid was removed by coevaporation with toluene, to yield a dark-red syrup. Without purification, the syrup in M H_2SO_4 was heated under reflux for 4 h. The acid was neutralized with $BaCO_3$, the suspension filtered, and the filtrate evaporated to a dark-brown syrup which was purified by flash chromatography using solvent *g*. Pure compound **4** was obtained as a syrup (10.35 g); $[\alpha]_D$ −29.8° (*c* 2.0, chloroform).

Compound **4** in anhydrous pyridine (50 mL) was cooled to 0°, and acetic anhydride (50 mL) was added. The solution was stirred overnight at room temperature; t.l.c. then showed the formation of a faster-moving compound, R_F 0.66 in solvent *d*. Aqueous workup gave compound **5** as a syrup (11.5 g, 72%); $[\alpha]_D$ −40° (*c* 0.5, chloroform), which was used without purification. For analysis, compound

5 (200 mg) was purified by chromatography on a column of silica gel using solvent *b*.

Anal. Calc. for $C_{15}H_{22}O_8$: C, 54.54; H, 6.66. Found: C, 54.92; H, 6.74.

3-O-Acetyl-4-O-allyl-1,2-O-(1-methoxyethylidene)-β-L-rhamnopyranose (**7**). — Compound **5** (6.78 g) was dissolved in dichloromethane (6 mL), acetic acid (7.5 mL) was added, and the solution was cooled to 0°. A 32% solution of hydrogen bromide in acetic acid (15 mL) was added dropwise during 15 min. After 1.5 h, t.l.c. in solvent *d* showed the disappearance of the starting material, formation of a faster-moving component of R_F 0.43, and traces of a decomposition product. The mixture was diluted with dichloromethane, successively washed extensively with cold water (3 × 75 mL), cold sodium hydrogencarbonate solution (3 × 50 mL), and water (2 × 50 mL), dried (anhydrous sodium sulfate), and evaporated, to afford a light-brown syrup (5 g, 69.5%). 2,6-Lutidine (12 mL) was added, followed by anhydrous methanol (2 mL). A white precipitate appeared after 1 h. The mixture was continuously stirred for 48 h, dichloromethane (12 mL) was added, and stirring was continued for an additional 16 h. Filtration, dilution of the filtrate, with ethyl acetate (10 mL), washing with cold water, drying, and evaporation yielded a syrupy residue (4 g, 91%). A small portion was purified for analytical purposes in an alumina column, using solvent *d*; $[\alpha]_D$ +30° (*c* 0.51, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.95 (m, 1 H, -C*H*=), 5.35 (d, 1 H, $J_{1,2}$ 2.4 Hz, H-1), 5.3–5.0 (m, 3 H, =CH_2 and H-3), and 4.54 (dd, 1 H, $J_{2,3}$ 4.0 Hz, H-2).

4-O-Allyl-1,2-O-(1-methoxyethylidene)-3-O-methyl-β-L-rhamnopyranose (**9**). — A solution of compound **7** (4 g) in anhydrous methanol (15 mL) was treated with freshly prepared, ammonia-saturated methanol (20 mL), the solution kept for 48 h at room temperature, and evaporated to a syrupy residue, and this used directly for the next step. A solution of the residue in dry tetrahydrofuran (20 mL) was cooled to 0°, and a suspension of sodium hydride (1 g) in tetrahydrofuran (5 mL) was added. The mixture was stirred for 2 h, cooled to 0°, methyl iodide (1 mL) added, and stirring continued overnight at room temperature. Methanol was added to decompose the excess of sodium hydride, the solvents were evaporated, and the residue was partitioned between water and chloroform. The chloroform phase was dried, and evaporated, and the syrupy residue was purified by column chromatography using alumina (basic) and solvent *b*. Compound **9** was obtained as a syrup (4 g, 95%); R_F 0.32, solvent *b*; $[\alpha]_D$ +20.36° (*c* 2.45, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.95 (m, 1 H, –C*H*=), 5.4 (d, 1 H, $J_{1,2}$ 2.4 Hz, H-1), 5.3–5.2 (m, 2 H, =CH_2), 4.6 (dd, 1 H, $J_{2,3}$ 4.0 Hz, H-2), 3.55–3.25 (2 s and m, ring C*H* and 2 CH_3O), 1.7 (s, 3 H, CCH_3), and 1.3 (d, 3 H, *J* 6.0 Hz, H-6); ^{13}C-n.m.r. ($CDCl_3$): δ 134.6 (–*C*H=), 116.0 (=*C*H_2), 97.0 (C-1), 81.1 (C-4), 78.6 (C-3), 76.0 (–O*C*H_2CH=), 73.4 (C-2), 69.86 (C-5), 57.2 (O*C*H_3), 49.2 (CO*C*H_3), 23.8 (C*C*H_3), and 17.4 (C-6).

Anal. Calc. for $C_{13}H_{22}O_6$: C, 56.90; H, 8.02. Found: C, 56.40; H, 7.85.

8-(Methoxycarbonyl)octyl O-(2-O-acetyl-4-O-allyl-3-O-methyl-α-L-rhamnopyranosyl)-(1→2)-4-O-benzyl-3-O-methyl-α-L-rhamnopyranoside (**12**). — To compound **9** (2 g, 7.3 mmol) in anhydrous dichloromethane (12 mL) was added chlorotrimethylsilane (2.5 mL). The mixture was heated under reflux for 2 h; t.l.c. at this

stage showed the disappearance of the starting material, and formation of a faster-moving component of R_F 0.51 in solvent *d*, which was ~95% pure according to t.l.c. The solvent was evaporated, and the excess of the reagent was removed by coevaporation with benzene. The syrupy 2-*O*-acetyl-4-*O*-allyl-3-*O*-methyl-α-L-rhamnopyranosyl chloride (**10**) was used directly for condensation.

To compound **11** (ref. 5; 0.9 g, 2.1 mmol) in anhydrous dichloromethane (12 mL) were added silver triflate (1.125 g, 4.37 mmol) and 1,1,3,3-tetramethylurea (1.6 mL), and the mixture was stirred, and cooled to −70° in an inert atmosphere. A solution of compound **10** (1.4 g, 5.02 mmol) in dichloromethane (9 mL) was slowly added to the mixture during 15 min (with a gastight syringe). The mixture was allowed to warm gradually overnight. Filtration followed by washing of the organic layer with sodium hydrogencarbonate solution (3 × 15 mL), gave on evaporation a syrup, which was purified by column chromatography using solvent *e*. The pure disaccharide was obtained as a syrup (1.1 g, 70%); $[\alpha]_D^{25}$ −40.3° (*c* 2.4, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 7.2 (m, 5 H, aromatic), 5.9 (m, 1 H, –C*H*=), 5.4 (dd, 1 H, $J_{2',3'}$ 4 Hz, H-2'), 5.3–5.1 (m, 2 H, =CH_2), 4.98 (d, 1 H, $J_{1',2'}$ 1.6 Hz, H-1'), 4.8–4.6 (q, 2 H, CH_2Ph), 4.7 (d, 1 H, $J_{1,2}$ 1.4 Hz, H-1), 3.7 (s, 3 H, COOCH_3), 3.42, 3.41 (2 s, 6 H, 2 CH_3O), 2.3 (t, 2 H, CH_2CO), 2.1 (s, 3 H, CH_3CO), and 1.7–1.2 [m, 18 H, H-6,6', and (CH_2)$_6$].

Anal. Calc. for $C_{36}H_{56}O_{12}$: C, 63.52; H, 8.23. Found: C, 63.50; H, 8.00.

8-(Methoxycarbonyl)octyl O-(4-O-allyl-3-O-methyl-α-L-rhamnopyranosyl)-(1→2)-4-O-benzyl-3-O-methyl-α-L-rhamnopyranoside (**13**). — The disaccharide **12** (1.2 g) in anhydrous methanol (30 mL) was cooled to 0°, and dry sodium methoxide (30 mg) was added. After stirring the mixture for 2 h at 0°, it was warmed to room temperature and stirred overnight. T.l.c. in solvent *d* showed the disappearance of the starting material and the formation of a slower-moving component, R_F 0.29. The solution was decationized with Dowex 50 (H^+) resin, and the solution evaporated to a syrup (1.0 g); $[\alpha]_D$ −70.7° (*c* 0.20, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 7.3 (m, 5 H, aromatic), 5.9 (m, 1 H, –C*H*=), 5.3 (m, 2 H, =CH_2), 5.2 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.9–4.5 (q, 2 H, CH_2Ph), 4.7 (d, 1 H, $J_{1,2}$ 1.4 Hz, H-1), 3.7 (s, 3 H, COOCH_3), 3.5 (2 s, 6 H, 2 CH_3O), and 2.4 (bs, 1 H, D_2O exch., O*H*); ^{13}C-n.m.r. (CD_3OD): δ 103.0 (C-1'), 100.5 (C-1), 83.0 (C-4), 82.4 (C-3'), 81.3 (C-4'), 80.9 (C-3), 75.7 (C-2), 75.5 (OCH_2Ph), 74.6 (–OCH_2CH=), 69.2, 69.0 (C-5,5'), 68.6 (2 C, OCH_2, C-2'), 59.1, 58.2, 51.2 (3 OCH_3), 18.3, and 18.2 (C-6,6').

Anal. Calc. for $C_{34}H_{54}O_{11}$: C, 63.94; H, 8.46. Found: C, 63.63; H, 8.58.

8-(Methoxycarbonyl)octyl O-(4-O-allyl-2,3-di-O-methyl-α-L-rhamnopyranosyl)-(1→2)-4-O-benzyl-3-O-methyl-α-L-rhamnopyranoside (**14**). — *Method A*. To disaccharide **13** (0.5 g) in anhydrous dichloromethane (5 mL) were added 2,6-di-*tert*-butylpyridine (2 mL) and methyl triflate (1 mL), and the solution was heated for 3 h at 80°. The dark-red product was purified by dry-column chromatography, and compound **14** was isolated as a syrup, R_F 0.41 in solvent *d*; $[\alpha]_D$ −81.4° (*c* 0.5, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.85 (m, 1 H, –C*H*=), 5.2 (m, 2 H, =CH_2),

5.02 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.8–4.5 (q, 2 H, CH_2Ph), 4.6 (d, 1 H, $J_{1,2}$ 1.4 Hz, H-1), 4.3 (dd, 1 H, $J_{2,3}$ 4.0 Hz, H-2), 3.6 (s, 3 H, $COOCH_3$), 3.4 (3 s, 9 H, 3 CH_3O), and 2.2 (t, 2 H, CH_2CO); ^{13}C-n.m.r. (CD_3OD): δ 100 (C-1,1'), 83.1 (C-4), 82.3 (C-3'), 81.2 (C-4'), 81.1 (C-3), 78.4 (C-2'), 75.9 (C-2), 75.8 (OCH_2Ph), 74.8 ($OCH_2CH=$), 69.3, 68.9 (C-5,5'), 68.5 (OCH_2), 59.1, 58.2, 57.9 (3 CH_3O), 51.9 ($COOCH_3$), 18.4, and 18.3 (C-6,6').

Method B. A solution of compound **13** (312 mg, 489 μmol) in dry tetrahydrofuran (THF; 5 mL) was cooled to 0°, and a suspension of sodium hydride (15 mg, 625 μmol) in THF (1 mL) was added. The mixture was stirred for 3 h at room temperature, cooled to 0°, methyl iodide (1.5 mL) was added, and stirring was performed overnight at room temperature. T.l.c. in solvent *d* then showed the emergence of a faster-moving component, R_F 0.4. Methanol was added, the solvents evaporated, and a solution of the residue in chloroform was washed with water, dried (magnesium sulfate), and evaporated to a syrup (0.310 g, 90%). The chemical analysis and physical constants of this compound were identical to those of compound **14** prepared by Method A.

Anal. Calc. for $C_{35}H_{56}O_{11}$: C, 64.41; H, 8.50. Found: C, 64.49; H, 8.31.

8-(Methoxycarbonyl)octyl O-(2,3-di-O-methyl-α-L-rhamnopyranosyl)-(1→2)-4-O-benzyl-3-O-methyl-α-L-rhamnopyranoside (**15**). — To compound **14** (1 g) in 7:3:1 ethanol–benzene–water (25 mL) were added 1,4-diazabicyclo[2.2.2]octane (0.5 g) and Wilkinson catalyst (0.1 g). The mixture was heated under reflux overnight and evaporated to a dark-yellow residue; a solution of this in chloroform was successively washed with water, cold M hydrochloric acid, aqueous sodium hydrogencarbonate, and water, dried, and evaporated. To a concentrated solution of the residue in 1:1 acetone–water (17 mL) was added mercuric oxide (350 mg), followed by a solution of mercuric chloride (350 mg) in 9:1 acetone–water (400 μL). The suspension was stirred for 1 h at room temperature, the solids filtered off, and the filtrate evaporated. A solution of the residue in chloroform was washed with water, aqueous potassium iodide, and water, dried, and concentrated. Column chromatography (solvent *i*) gave pure compound **15** (750 mg, 80%); $[\alpha]_D$ −43.8° (*c* 0.8, methanol); ^{1}H-n.m.r. ($CDCl_3$): δ 5.1 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.9–4.6 (q, CH_2Ph), 4.72 (d, 1 H, $J_{1,2}$ 1.0 Hz, H-1), 3.66 (s, 3 H, $COOCH_3$), 3.54, 3.51, 3.50 (3 s, 9 H, 3 CH_3O), 2.4 (t, 2 H, CH_2CO), and 1.66–1.2 [m, 18 H, H-6,6' and $(CH_2)_6$]; ^{13}C-n.m.r. (CD_3OD): δ 100.4, 100.2 (C-1',1), 83.2 (C-4), 82.1 (C-3'), 81.4 (C-3), 78.3 (C-2'), 75.9 (C-2), 75.8 (OCH_2Ph), 73.1 (C-4'), 70.5 (C-5'), 69.1 (C-5), 59, 58.2, 57.9 (3 CH_3O), 51.8 ($COOCH_3$), and 18.3 and 18 (C-6,6').

Anal. Calc. for $C_{32}H_{52}O_{11}$: C, 62.74; H, 8.49. Found: C, 63.40; H, 8.45.

8-(Methoxycarbonyl)octyl O-(2,4-di-O-3,6-di-O-methyl-β-D-glucopyranosyl)-O-(1→4)-O-(2,3-di-O-methyl-α-L-rhamnopyranosyl)-(1→2)-3-O-methyl-α-L-rhamnopyranoside (**17**). — To a well stirred solution of compound **15** (0.800 g, 1.3 mmol) in anhydrous dichloromethane (7 mL) containing mercuric cyanide (0.300 g, 1.18 mmol), mercuric bromide (0.120 g), and powdered molecular sieves (1.5 g), was added dropwise a solution of freshly prepared 2,4-di-*O*-acetyl-3,6-di-*O*-methyl-

D-glucopyranosyl bromide[5] (**16**) (0.900 g, 2.53 mmol) in dry dichloromethane (5 mL). The mixture was stirred for 16 h at room temperature under an inert atmosphere. T.l.c. (solvent *f*) then showed the formation of a new component, R_F 0.6. The solids were removed by filtration and washed with dichloromethane. The filtrate and washings were combined, successively washed with water, M potassium bromide, and water, dried, and evaporated. Compound **17** was purified by column chromatography in solvent *f*; yield 800 mg (69%); $[\alpha]_D$ −41.6° (*c* 0.3, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.2 (m, 2 H, H-2″,4″), 4.9 (s, 1 H, H-1′), 4.75 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1), 4.45 (d, 1 H, $J_{1'',2''}$ 7.81 Hz, H-1″), 3.65–3.45 (bs, 18 H, 6 CH_3O), 2.25 (t, 2 H, CH_2CO), and 2.15 (s, 6 H, 2 CH_3CO).

Anal. Calc. for $C_{44}H_{70}O_{18}$: C, 59.60; H, 7.90. Found: C, 59.20; H, 8.05.

8-(Methoxycarbonyl)octyl O-(3,6-di-O-methyl-β-D-glucopyranosyl)-(1→4)-O-(2,3-di-O-methyl-α-L-rhamnopyranosyl)-(1→2)-4-O-benzyl-3-O-methyl-α-L-rhamnopyranoside (**18**). — Compound **17** (0.500 g) in anhydrous methanol (10 mL) was cooled to 0°, and dry sodium methoxide (50 mg) was added. After being stirred for 2 h at 0°, the solution was warmed to room temperature and kept overnight. Decationization followed by filtration and evaporation gave a homogeneous syrup (350 mg, 77%); $[\alpha]_D$ −44.0° (*c* 0.54, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.0 (bs, 1 H, H-1′), 4.7 (s, 1 H, H-1), 4.5 (q, 2 H, CH_2Ph), 4.4 (d, 1 H, $J_{1'',2''}$ 7.5 Hz, H-1″), 3.8–3.3 (bs, 18 H, 6 MeO), 2.2 (t, 2 H, CH_2CO), and 1.6–1.1 [m, 18 H, H-6,6′ and $(CH_2)_6$]; ^{13}C-n.m.r. (CD_3OD): δ 105.0 (C-1″), 100.2 (C-1,1′), 87.6 (C-3″), 83.1 (C-4), 82.1 (C-3′), 81.3 (C-3), 79.8 (C-4′), 77.8 (C-2′), 76.7 (C-5″), 75.9 (C-2), 75.7 (C-2″), 75.5 (OCH_2Ph), 73.5 (C-6″), 71.2 (C-4″), 69.1, 69 (C-5,5′), 68.6 (OCH_2), 60.7, 59.7, 59.1, 58.2, 57.4 (5 CH_3O), 51.8 ($COOCH_3$), and 18.4 and 18.2 (C-6,6′).

Anal. Calc. for $C_{40}H_{66}O_{16}$: C, 59.85; H, 8.22. Found: C, 59.41; H, 8.02.

8-(Methoxycarbonyl)octyl 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-rhamnopyranoside (**19**). — Hydrogenolysis of trisaccharide **18** (200 mg) in ethanol (5 mL) containing acetic acid (200 μL) for 16 h at room temperature under atmospheric pressure using 10% palladium-on-charcoal (50 mg) gave compound **19** as a syrup (148 mg, 85%); $[\alpha]_D$ −39.2 (*c* 0.3, methanol); ^{1}H-n.m.r. ($CDCl_3$): δ 5.01 (d, 1 H, $J_{1',2'}$ 1.2 Hz, H-1′), 5.0 (m, 2 H, H-2″,4″), 4.7 (d, 1 H, $J_{1,2}$ 1 Hz, H-1), 4.4 (d, 1 H, $J_{1'',2''}$ 7.7 Hz, H-1″), 3.60 (s, 3 H, $COOCH_3$), 3.52–3.51 (5 s, 5 MeO), 2.3 (t, 2 H, CH_2CO), and 1.7–1.1 [m, 18 H, H-6,6′, and $(CH_2)_6$]; ^{13}C-n.m.r. (CD_3OD): 105.1 (C-1″), 100.3, 100.2 (C-1,1′), 87.6 (C-3″), 82.5 (C-3), 82.1 (C-3′), 79.8 (C-4′), 77.8 (C-2′), 76.8 (C-5″), 75.8 (C-2), 75.5 (C-2″), 73.5 (C-4), 73.2 (C-6″), 71.3 (C- 4″), 68.7 (OCH_2), 60.7, 59.7, 59.0, 58.3, 57.3 (5 CH_3O), and 51.8 ($COOCH_3$).

8-Carbazoyloctyl 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-rhamnopyranoside (**20**). — The deblocked trisaccharide **19** (100 mg) was dissolved in ethanol (5 mL), and 85% hydrazine hydrate (400 μL) was added. The solution was stirred for 48 h, and evaporated, and the excess of hydrazine was removed by codistillation with water. Column chromatography on Sephadex LH 20 with solvent *h* as the eluant gave

pure hydrazide **20** (85 mg, 86%); R_F 0.36, solvent *j*; $[\alpha]_D$ −20° (*c* 1.0, chloroform).

Anal. Calc. for $C_{32}H_{60}N_2O_{15}$: C, 53.90; H, 8.42; N, 3.90. Found: C, 53.40; H, 8.60; N, 3.40.

O-(3,6-Di-O-methyl-β-D-glucopyranosyl)-(1→4)-O-(2,3-di-O-methyl-α-L-rhamnopyranosyl)-(1→2)-O-(3-O-methyl-α-L-rhamnopyranosyl)-(1→9)-oxynonanoyl-BSA (natural trisaccharide-octyl-BSA; NT-O-BSA) (**21**). — The freeze-dried hydrazide **20** (40 mg, 56 μmol) was dissolved in dry *N,N*-dimethylformamide (1 mL), 3.6M HCl–1,4-dioxane (150 μL) was added, and the solution was cooled to −30°. *tert*-Butyl nitrite (1:10 dilution in *N,N*-dimethylformamide; 225 μL) was added, and the solution was stirred for 30 min at −30°. T.l.c. in solvent *j* showed the disappearance of the starting material, and formation of a new component having R_F 0.63. The nitrous acid was neutralized with 0.5M sulfamic acid (225 μL). The cold acyl azide solution was added dropwise to a stirred solution of BSA (40 mg, 0.58 μmol) in 4 mL of 0.08M $Na_2B_4O_7$ and 0.3M $KHCO_3$ (pH 9.2), at 0°. The solution became cloudy after 30 min. Stirring was continued overnight at 4°. The solution was dialyzed against four changes of water in an ultrafiltration cell (Amicon Corp., Danvers, MA) equipped with a PM-10 membrane. The retentate was subjected to gel filtration (Sephadex G-75 in phosphate-buffered saline). The protein-positive fractions (280 nm) were analyzed for hapten content by carbohydrate assay[5], using L-rhamnose as the standard. An incorporation of 40–45 mol of hapten per mol of BSA was achieved.

O-(3,6-Di-O-methyl-β-D-glucopyranosyl)-(1→4)-O-(2,3-di-O-methyl-α-L-rhamnopyranosyl)-(1→2)-O-(3-O-methyl-α-L-rhamnopyranosyl-(1→4′)-oxy-(3-phenylpropanoyl)-BSA (natural trisaccharide-phenylpropanoyl-BSA; NT-P-BSA) (**22**). — Full details of the synthesis of NT-P-BSA have been described[9]. Briefly, benzyl 4-*O*-benzyl-3-*O*-methyl-α-L-rhamnopyranoside was selectively allylated at OH-2 followed by isomerization of the allyl to a 1-propenyl group with potassium *tert*-butoxide. The isomerized rhamnopyranoside was treated with 3M trifluoroacetic acid, and the sugar acetylated with acetic anhydride–pyridine, to give 1,2-di-*O*-acetyl-4-*O*-benzyl-3-*O*-methyl-α,β-L-rhamnopyranose. The α-acetate was isolated, and converted into the corresponding 1-bromide by using titanium tetrabromide, and the bromide coupled with methyl 3-(4-hydroxyphenyl)propanoate, to give 4-(2-methoxycarbonylethyl)phenyl 2-*O*-acetyl-4-*O*-benzyl-3-*O*-methyl-α-L-rhamnopyranoside in a yield of ~55%. The 2-*O*-acetyl group was then removed with sodium methoxide.

The condensation of this reducing-end derivative with the relatively stable glycosyl donor *O*-(2,4-di-*O*-acetyl-3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranosyl chloride, in the presence of 1,1,3,3-tetramethylurea and silver triflate, gave the blocked trisaccharide in ~35% yield. On deacetylation and debenzylation, this gave 4-(2-methoxycarbonylethyl)phenyl *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-rhamnopyranoside). This glycoside was converted into the acylazide *via* the hydrazide, and the product was coupled to BSA;

a coupling ratio of haptens to protein of 40:1 was achieved.

Poly(acrylamide) gel electrophoresis and immunoblotting of neoglycoconjugates. — The neoglycoconjugates (10–20 μg) were dissolved in 20 μL of denaturing buffer, and 1 μL was applied to slab gels (3 × 4 cm) according to the method of Laemmli and Favre[32]. The separation gels were composed of 10% of acrylamide cross-linked with 0.8% of bis(acrylamide). A 6% acrylamide stacking gel was layered above the separating gel. After electrophoresis at 10 mA/gel at constant current, gels were stained with Coomassie Blue, or the bands were transferred electrophoretically to nitrocellulose sheets for antibody binding as described previously[33]. Electrophoresis was performed in 25mM Tris, 192mM glycine, and 20% (v/v) CH_3OH (pH 8.2), in a Bio-Rad Trans-Blot apparatus[33].

Preparation of monoclonal antibody to PGL-I. — An immunogenic sample of PGL-I was prepared by mixing whole armadillo-derived *M. leprae* (10 mg in 3 mL of H_2O) and PGL-I (5 mg in 2 mL of H_2O) by sonication. A portion (0.5 mL) of the resulting preparation was mixed with an equal volume of Freund's incomplete adjuvant (Sigma, St. Louis, MO) by using a double-hubbed emulsifying needle. A 0.1-mL aliquot of the antigen–adjuvant mixture was injected into the peritoneal cavity of five BALB/c mice. Mice were given a booster injection three weeks later, and were bled for testing nine days after the booster injection. The mouse showing the highest antibody response to PGL-I in plate-ELISA[28] was further inoculated intravenously, this time with the antigen preparation (0.1 mL) only. Three days later, the mouse was sacrificed; spleen cells were harvested, and fused with SP2/O-Ag14 cells[34]. Culture supernatant liquors from wells containing growing cells were tested for antibody production against PGL-I using ELISA[28]. Cells producing anti-PGL-I antibodies were cloned twice. A cell line producing a high titer of anti-PGL-I antibodies was established[34].

Serology. — Indirect ELISA, as described by Voller *et al.*[29] with important modifications (see ref. 28, and later), was used to detect IgM antibodies to PGL-I and the neoantigens. Briefly, 50 μL of a suspension of PGL-I (2 μg/mL), or a solution of the neoantigens (50–100 ng of L-rhamnose equivalent/mL) prepared as described[28], was applied to wells of U-bottomed microtiter plates (Dynatech Laboratories, Alexandria, VA), and incubated overnight at 37° in a moist chamber. Unbound antigen was removed with phosphate-buffered saline, pH 7.4, containing 0.05% of Tween 20 (PBST); PBST containing 0.5% of BSA (100 μL) was added to wells, and the plates were incubated for 1 h at 37°. When PGL-I was used as antigen, Tween 20 was omitted from the PBST. After removal of blocking solution, 50 μL of human serum diluted 1:300 in PBS containing 5% of normal goat serum (NGS) was added to wells, and these were incubated for 90 min at 37°. After further washing, 50 μL of affinity-purified, peroxidase-conjugated, goat anti-human IgM (Calbiochem, San Diego, CA) diluted 1:5000 in PBST–5% NGS was added, and incubated for 1 h at 37°. Finally, after another washing, 50 μL of substrate composed of *o*-phenylenediamine (0.4 mg/mL) and 30% H_2O_2 (0.4 μL/mL) in citrate–phosphate buffer, pH 5.0, was added, and the plates were incubated for 15–20 min

at room temperature in a dark chamber. The reaction was stopped with 50 μL of 1.25M H_2SO_4, and the absorbance was read at 490 nm. The concentration of the individual antigen was adjusted by block titration against a positive control serum from a lepromatous leprosy patient, to obtain an absorbance value (A_{490}) of 1.200.

ACKNOWLEDGMENTS

The authors acknowledge research support from the National Institute of Allergy and Infectious Diseases (Contract NO1-AI-52582 and Grant AI-24154). We thank R. H. Gelber, M. D., for sera and information on clinical status of patients; Don Dick and Susie Miller, respectively, for mass spectrometric and n.m.r.-spectral analyses; Carol Marander for the graphics; and Marilyn Hein for preparation of the manuscript.

REFERENCES

1 S. W. HUNTER, T. FUJIWARA, AND P. J. BRENNAN, *J. Biol. Chem.*, 257 (1982) 15,072–15,078.
2 T. FUJIWARA, S. W. HUNTER, S.-N. CHO, G. O. ASPINALL, AND P. J. BRENNAN, *Infect. Immun.*, 43 (1984) 245–252.
3 S.-N. CHO, T. FUJIWARA, S. W. HUNTER, T. H. REA, R. H. GELBER, AND P. J. BRENNAN, *J. Infect. Dis.*, 150 (1984) 311–322.
4 D. CHATTERJEE, J. T. DOUGLAS, S.-N. CHO, T. H. REA, R. H. GELBER, G. O. ASPINALL, AND P. J. BRENNAN, *Glycoconjugate J.*, 2 (1985) 187–208.
5 D. CHATTERJEE, S.-N. CHO, P. J. BRENNAN, AND G. O. ASPINALL, *Carbohydr. Res.*, 156 (1986) 39–56.
6 S.-N. CHO, D. CHATTERJEE, AND P. J. BRENNAN, *Am. J. Trop. Med.*, 35 (1986) 167–172.
7 S.-N. CHO, D. CHATTERJEE, AND P. J. BRENNAN, *Abstr. Pap. Annu. Meet. ASM, 86th*, Washington, D.C., p. 120, No. U-6; L. SHENGKAI, L. FUTIAN, AND P. J. BRENNAN, unpublished observations.
8 T. FUJIWARA, S. IZUMI, AND P. J. BRENNAN, *Agric. Biol. Chem.*, 49 (1985) 2301–2308.
9 T. FUJIWARA AND S. IZUMI, *Agric. Biol. Chem.*, 51 (1987) 2539–2547.
10 S. J. BRETT, S. N. PAYNE, J. GIGG, P. BURGESS, AND R. GIGG, *Clin. Exp. Immunol.*, 64 (1986) 476–483.
11 R. GIGG, S. N. PAYNE, AND R. CONANT, *J. Carbohydr. Chem.*, 2 (1983) 207–223.
12 G. AGUADO SANCHEZ, A. MALIK, C. TOUGNE, P. H. LAMBERT, AND H. D. ENGERS, *Lepr. Rev.*, 57 (1986) 83–93.
13 H. GAYLORD AND P. J. BRENNAN, *Annu. Rev. Microbiol.*, 41 (1987) 645–675.
14 J. GIGG, R. GIGG, S. PAYNE, AND R. CONANT, *Chem. Phys. Lipids*, 38 (1985) 299–307.
15 T. FUJIWARA, G. O. ASPINALL, S. W. HUNTER, AND P. J. BRENNAN, *Carbohydr. Res.*, 163 (1987) 41–52.
16 R. U. LEMIEUX, D. R. BUNDLE, AND D. A. BAKER, *J. Am. Chem. Soc.*, 97 (1975) 4071–4086.
17 H. B. BORÉN, G. EKBORG, K. EKLIND, P. J. GAREGG, Å. PILOTTI, AND C.-G. SWAHN, *Acta Chem. Scand.*, 27 (1973) 2639–2644.
18 D. R. BUNDLE AND S. JOSEPHSON, *Can. J. Chem.*, 57 (1979) 662–668.
19 A. LIPTÁK, P. NANASI, A. NESZMÉLYI, AND H. WAGNER, *Tetrahedron*, 36 (1979) 1261–1268.
20 J. S. BRIMACOMBE, *Methods Carbohydr. Chem.*, 6 (1972) 376–378.
21 S. J. JOSEPHSON AND D. R. BUNDLE, *J. Chem. Soc., Perkin Trans. 1*, (1980) 297–301.
22 S. HANESSIAN AND J. BANOUB, *Carbohydr. Res.*, 53 (1977) c13–c16.
23 D. B. YOUNG, S. R. KHANOLKAR, L. L. BARG, AND T. M. BUCHANAN, *Infect. Immun.*, 43 (1984) 183–188.
24 V. MEHRA, P. J. BRENNAN, E. RADA, J. CONVIT, AND B. R. BLOOM, *Nature*, 308 (1984) 194–196.
25 S.-N. CHO, S. W. HUNTER, R. H. GELBER, T. H. REA, AND P. J. BRENNAN, *J. Infect. Dis.*, 153 (1986) 560–569.

26 D. S. RIDLEY AND W. H. JOPLING, *Int. J. Lepr.*, 34 (1966) 255–273.
27 R. H. GELBER, in H. CONN (Ed.), *Current Therapy*, W. B. Saunders, Philadelphia, 1978, p. 32.
28 S.-N. CHO, D. L. YANAGIHARA, S. W. HUNTER, R. H. GELBER, AND P. J. BRENNAN, *Infect. Immun.*, 41 (1983) 1077–1083.
29 A. VOLLER, D. E. BIDWELL, AND A. BARTLETT, *Procedures*, in *The Enzyme Linked Immunosorbent Assay (ELISA)*, Dynatech Laboratories, Inc., Alexandria, VA, 1979, pp. 23–125.
30 D. D. PERRIN, W. L. ARMAREGO, AND D. R. PERRIN (Eds.), *Purification of Laboratory Compounds*, 2nd edn., Pergamon Press, London, 1980.
31 W. C. STILL, M. KAHN, AND A. MITRA, *J. Org. Chem.*, 43 (1978) 2933–2935.
32 U. K. LAEMMLI AND M. FAVRE, *J. Mol. Biol.*, 80 (1973) 575–599.
33 S. W. HUNTER, H. GAYLORD, AND P. J. BRENNAN, *J. Biol. Chem.*, 261 (1986) 12,345–12,351.
34 L. HUDSON AND F. C. HAY, *Practical Immunology*, 2nd edn., Blackwell Scientific Publications, London, 1980, pp. 303–327.