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The carbohydrate-containing antigens of *Mycobacterium leprae*

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The overt antigenicity of *Mycobacterium leprae,* as for all mycobacteria, is dominated by carbohydrate-containing entities. We now know sufficient of the immunochemistry of *M. leprae* to realize that its carbohydrate-based antigens are relatively simple; perhaps, the *in vivo* setting of *M. leprae,* prior to its isolation, ensures limited autolysis. The dominant carbohydrate-containing epitopes of *M. leprae* are contained within but three classes of structures: the phenolic glycolipids, the lipoarabinomannan and the insoluble cell wall skeleton. Our work over the past few years has addressed each of these complexes and herein is presented a brief review of our studies.

The glycolipid antigens of *M. leprae*

Most mycobacteria are endowed with large quantities of glycolipids that contain within them small oligosaccharides of sufficient antigenicity to evoke antibodies of such exquisite specificity as to allow unequivocal identification of species or subspecies.

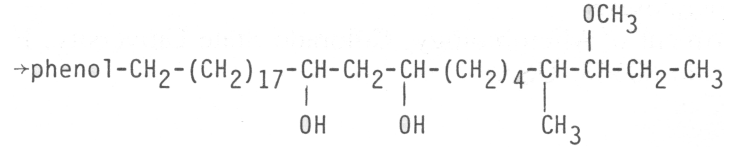
In the course of establishing this principle, investigators in our laboratory have examined many members of the *Mycobacterium* genus, and out of these endeavors has emerged the realization that the specific immunoreactive glycolipids of mycobacteria are of three classes: the C-mycoside glycopeptidolipids; the trehalose-containing lipooligosaccharides; and the phthiocerol-containing phenolic glycolipids. The structures and antigenicity of these three groups have been recently reviewed (1, 2, 3).

The recognition of a distinct class of glycolipid antigens, the phenolic glycolipids, arose from a deliberate quest in *M. leprae* for species-specific lipid antigens of the type present in nontuberculous mycobacteria. Initial studies exposed a partially purified lipid fraction that produced distinct lines of precipitation with antisera from two lepromatous leprosy patients and from an infected armadillo, and there was no reaction to sera from patients with tuberculosis or an *M. avium* infection (4). The activity in the lipid fraction was unaffected by mild alkali, suggesting that it was related to one of the «mycoside» classes (4). An important development in terms of isolating the glycolipid in quantities sufficient for chemical analysis was the realization that it occurred in enormous quantities in tissues surrounding foci of infections and, indeed, corresponded to the «electron transparent zone» or capsule often evident in lepromatous leprosy lesions (5). Infrared and NMR spectroscopy showed that the glycolipid was closely related to «mycoside A» from *M. kansasii* (6), clearly indicating that it was of the triglycosylphenolic phthiocerol diester class. However, there was the crucial difference in that the product from *M. leprae* contained a unique combination of sugars that were identified by gas chromatography/mass spectrometry and by demethylation to expose the parental sugar, as 3,6-di-*O*-methylglucose, 2,3-di-*O*-methylrhamnose, and 3-*O*-methyl-

rhamnose (5). Subsequently (7), the structure of the oligosaccharide entity was determined by partial acid hydrolysis, permethylation, ^{1}H-NMR, and ^{13}C-NMR as

3,6-di-*O*-methyl-β-D-glucopyranosy (1→4) 2,3-di-*O*-methyl-α-L-rhamnopyranosyl (1→2) 3-*O*-methyl-α-L-rhamnopyranosyl-(1→

Acid hydrolysis of the deacylated glycolipid yielded a phenolic phthiocerol «core», and mass spectrometry and proton NMR of the permethylated core established the following structure (7):



Combined gas-liquid chromatography/mass spectrometry showed three tetramethyl branched «mycocerosic» acids. C_{30}, C_{32}, and C_{34}, whose molecular weights (as methyl esters) were 466, 494, and 522, respectively; these are esterified to the hydroxyl functions of the branched glycolic chain. Thus the full structural details of the group-specific dimycocerosyl phenolic phthiocerol and the species-specific triglycosyl entity were established. To date, we have recognized and fully characterized three such phenol-phthiocerol triglycosides (7,8,9):

3,6-di-O-Me-β-D-Glcp(1→4)2,3-di-O-Me-α-L-Rhap(1→2)3-O-Me-α-L-Rhap →

Phenolic glycolipid-I (PGL-I)

3,6-di-O-Me-β-D-Glcp(1→4)3-O-Me-α-L-Rhap(1→2)3-O-Me-α-L-Rhap →

Phenolic glycolipid-II (PGL-II)

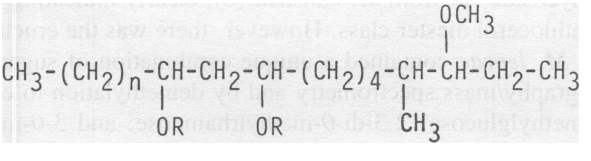
6-O-Me-β-D-Glcp(1→4)2,3-di-O-Me-α-L-Rhap(1→2)3-O-Me-α-L-Rhap →

Phenolic glycolipid-III (PGL-III)

PGL-II differs from PGL-I in the absence of an *O*-CH_3 group at the C-2 of the penultimate rhamnose, and PGL-III differs in the absence of an *O*-CH_3 group from C-3 of the distal glucose residue. Thus PGL-II and -III are probably minor catabolic or anabolic by-products of PGL-I.

Tarelli *et al.* (10) have recently published independent studies on the structure of the trisaccharide portion of PGL-I.

In addition, the nonphenylated, nonglycosylated dimycocerosyl-phthiocerol has been isolated in large quantities from infected armadillo tissue and characterized completely (8,11).



n = 16, 18

From the work of Young (12) and Izumi *et al.* (13), it is certain that phenolic glycolipid-I, at least, and the diacylphthiocerol, are also present in large quantities in human lepromas. Izumi *et al.* showed that phenolic glycolipid-I could be recovered in massive quantities from formalin-fixed livers of advanced lepromatous leprosy patients from the prechemotherapeutic era.

Initially, the antigenicity of PGL-I had been inferred because it was the only *M. leprae*-specific product obtained from the active preparation originally described (4). The untoward lipophilicity of highly purified PGL-I, as distinct from the impure preparation, prevented the use of several standard serological assays, and thus, for a while, its antigenicity was a moot question (5). Payne *et al.* (14) adopted the ploy of incorporating PGL-I into double-membrane liposomes, which could then diffuse in agarose gels, and it was demonstrated that such liposomes produced precipitates with undiluted sera from three patients with active lepromatous leprosy but no precipitates with sera from patients with tuberculoid leprosy, from mice heavily infected with *M. lepraemurium,* or with sera from patients with active pulmonary tuberculosis. On the other hand, we reasoned that ELISA protocols offered the greatest promise of a sensitive assay for antiglycolipid antibodies, in that, under the proper coating conditions, the glycolipid should orient itself on the solid substratum in such a manner as to render the diacylphthiocerol cryptic and the oligosaccharides more amenable for reaction. Indeed, the phenolic glycolipids reacted readily in ELISA with hyperimmune anti-*M. leprae* rabbit antiserum, to the extent that as little as 5 ng reacted with serum diluted 1:100 from intramuscularly immunized animals (7,8) However, these original ELISA conditions were not suitable for the routine diagnosis of human leprosy because sera from healthy humans gave abnormally high absorption values. A new set of sensitive ELISA conditions were developed that were more in accord with the extreme hydrophobicity of native PGL-I and the variable titer of anti-PGL-I antibodies in human leprosy sera (15). While it is difficult to attribute the success of this protocol to any particular parameter, the important features are: thorough sonication of the glycolipid in a high pH coating buffer to arrive at consistently small lipid vesicles; the deletion of detergents as blocking agents and as diluents and the inclusion of heterologous bovine serum albumin and homologous normal goat serum in buffer to prevent nonspecific binding of antibodies to the polystyrene substratum. The sensitivity of the assay was impressive; 2 μg/ml buffer (100 ng/well) produced an absorption value of 0.972 + 0.100 (triplicate) with pooled lepromatous sera diluted 1:300. With these vastly improved conditions, we have demonstrated that the antibody response in human sera to PGL-I is primarily IgM, to the extent of about 90 % of total measured immunoglobulins (IgG, IgM, and IgA), although this varies with the leprosy state (16).

Young and Buchanan (17) independently developed an ELISA in which the deacylated form of the phenolic glycolipid was used. Under their assay conditions, the intact glycolipid was considerably less active; however, under the conditions described by Brett *et al.* (18), which employed detergent, or the conditions of Cho *et al.* (15), using sonicated whole glycolipid, both the deacylated and native glycolipid were about equally active. Despite differences in protocol, source of sera, reagents, etc., there is considerable accord in the results reported from these independent studies: (1) antibodies directed against the glycolipid were seen in sera from leprosy patients but not sera from uninfected controls or patients infected with mycobacteria other than *M. leprae;* (2) approximately 96 % of lepromatous leprosy patients demonstrated antiglycolipid antibodies compared with about 50-60 % of tuberculoid patients; (3) treatment of lepromatous leprosy patients resulted in significantly lower antiglycolipid antibodies; (4) the antibody response distinguished between the phenolic glycolipid from *M. leprae* and the structurally related phenolic glycolipids from *M. bovis* and *M. kansasii;* (5) the antigenic determinant was recognized as within the specific trisaccharide

entity; the aglycon segment was devoid of activity; (6) serological assays based on the glycolipid seemed to have considerable potential for the specific diagnosis of leprosy. There were some differences in the results that cannot readily be explained by different protocols. For instance, Brett *et al.* (18) observed both high antiglycolipid IgM and IgG, whereas Cho *et al.* (15,16) and Young *et al.* (19) reported that the dominant human antibody response was IgM and that the IgG was probably not significant. In an effort to resolve this issue, Cho *et al.* (20) have recently isolated and separated IgG and IgM from patient sera, using ion-exchange column chromatography, and again demonstrated predominance of anti-glycolipid IgM.

Neoglycoproteins containing the glycosyl units of PGL-I

Previously, successful synthesis of the trisaccharide component of PGL-I (9) and the terminal disaccharide substituent of PGL-I and -III (9,21) had been described. Fujiwara *et al.* (9) had also shown that in an ELISA inhibition assay, only those oligosaccharides with the full complement of methoxyl groups at the nonreducing end were active in binding human leprosy anti-glycolipid IgM. Subsequently, they showed that the first generation of neoglycoproteins, those derived by reductive amination of the synthetic disaccharides, the *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→34)-(1-deoxy-2,3-di-*O*-methyl-L-rhamnitol)-lysyl-bovine serum albumin (Fig. 1A) only, was serologically active, whereas those based on the 3'-mono-*O*-Me-β-D-glucopyranoside or the nonmethylated β-D-glucopyranoside were virtually inactive (16).

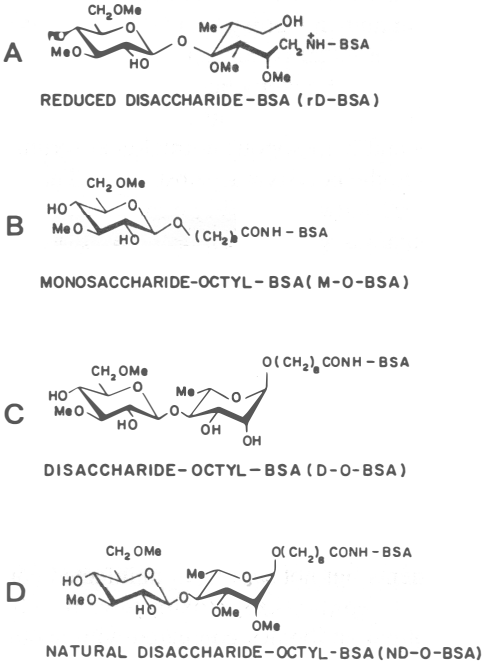


Figure 1.
Disaccharide-containing neoglycoproteins.

The fact that the 3,6-di-*O*-Me-β-D-glucopyranosyl-containing product of reductive amination was so highly active against lepromatous leprosy and showed excellent concordance with phenolic glycolipid I had itself indicated that only the terminal 3,6-di-*O*-methyl-β-D-glucopyranosyl was necessary for recognition of anti-glycolipid IgM. In addition, it had been shown that partially deglycosylated PGL-I, the product devoid of terminal di-*O*-methylglucose, had lost the majority of its ability to recognize IgM antibodies in human lepromatous leprosy serum (9) and murine monoclonal IgM (22) or monoclonal IgG antibodies (23,24). Thus, there was every rationale for the synthesis of neoglycoproteins based on the single sugar epitope. It was also imperative to arrive at a second generation of artificial antigens, since the first, those arrived at by reductive amination, showed considerable nonspecific binding to antibodies in normal individuals and those with tuberculosis (16).

Accordingly, a stratagem for the synthesis of neoglycoproteins suitable for the selective serodiagnosis of leprosy was developed in which synthetic 3,6-di-*O*-methyl-β-D-glucopyranose, the single epitope of phenolic glycolipid I from *Mycobacterium leprae,* was used (25). Condensation of 8-methoxycarbonyloctanol with the acetobromo derivative of 3,6-di-*O*-methylglucose gave 8-methoxycarbonyloctyl 2,4-di-*O*-acetyl-3,6-di-*O*-methyl-β-D-glucopyranoside in 65 % yield, and with absolute stereospecificity for the β anomer. The deacylated product was converted to the crystalline hydrazide and coupled to bovine serum albumin (also bovine gamma globulin and poly-*D*-lysine) *via* intermediate acylazide formation to produce the *O*-(3,6-di-*O*-methyl-β-D-glucopyranoyl)-(1→9)-oxynonanoyl-bovine serum albumin, the so-called monosaccharide-octyl-BSA (M-O-BSA) (Fig. 1B). The neoglycoproteins were highly sensitive in ELISA and emulated the specificity of the native glycolipid in analysis of sera from patients throughout the spectrum of leprosy and from different geographical regions.

A different synthetic approach (25) produced *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-(α-L-rhamnopyranosyl)-(1→9)-oxynonanoyl-BSA, the so-called disaccharide-octyl-BSA) (D-O-BSA) (Fig. 1C), which was also highly sensitive and specific for the serodiagnosis of leprosy, and the presence of the second sugar unit, similar to that in the native glycolipid but for the absence of *O*-methyl groups, provided a probe with greater felicity for the serological detection of tuberculoid leprosy. Thus, the results indicate that highly sensitive and specific antigen probes for the serodiagnosis of leprosy can be constructed based only on the terminal one or two sugars of phenolic glycolipid I, and the synthetic approach leads to the formation of haptens with absolute stereospecificity.

We have also finalized the synthesis of *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-*O*-(2,3-di-*O*-methyl-α-L-rhamnopyranosyl)-(1→9)-oxynonanosyl-BSA the natural disaccharide-octyl-BSA (ND-O-BSA) (26) (Fig. 1D). This was accomplished with both high yield and high stereospecificity by a series of modified Koenigs-Knorr and Helferich conditions.

The ND-O-BSA and those neoglycoconjugates described briefly above were tested initially in Colorado in ELISA against a variety of sera, leprosy and otherwise (26). All of the glycoconjugates proved to be highly reactive against lepromatous pooled sera, and showed good concordance with the native glycolipid in analysis of serum specimens of leprosy patients. The correlation coefficients (r) in absorbance between PGL-I and the glycoconjugates were from 0.753 to 0.909 ($p > 0.001$, Student t test).

Despite the good agreement (about 90 %) between the native glycolipid and the glycoconjugates in determining leprosy seropositivity or seronegativity, there was some evidence of discrepancy in the case of some glycoconjugates. Of 223 serum specimens assayed, nine (4 %) were positive to PGL-I, but negative to rD-BSA. Furthermore, in more than 15 serum samples, the reactivity against rD-BSA was less than half of that against PGL-I. This indicat-

ed that more of the native structure was required for optimal reaction with anti-PGL-I antibodies. Accordingly, we compared directly D-O-BSA and ND-O-BSA, each of which contains progressively more of the native structure. Two different sets of serum specimens were assayed. The results from the first study with 122 samples, from the Seton Medical Center, California, showed 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 to ND-O-BSA and 0.571 to D-O-BSA.

The study with the second group of leprosy patients from the Los Angeles Country-University of Southern California Medical Center also showed similar results to the first. ND-O-BSA gave 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. Thus, ND-O-BSA showed higher sensitivity in detecting anti-PGL-I antibodies than D-O-BSA. From both studies, however, it was consistently noticed that the tuberculoid class of leprosy patients had less antibody level than those at the lepromatous end. Among the lepromatous groups, patients with active disease or those treated for less than two years showed much higher antibodies than those with inactive disease or those subjected to prolonged treatment of more than two years.

Although both ND-O-BSA and D-O-BSA had excellent ability to react with anti-PGL-I antibodies, the slight advantage of ND-O-BSA over D-O-BSA was also demonstrated from a study with control groups. These consisted of 142 healthy controls, 66 tuberculosis patients, and 15 non-tuberculous mycobacterioses patients. 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.

As indicated above, there was always discrepancy among a minority of sera when native PGL-I and neoglycoconjugates were used. When some of these aberrant specimens were closely examined, certain trends emerged. Three serum specimens showed activity against PGL-I but no anti-PGL-I antibodies against any of the glycoconjugates, suggesting that more of the structure of the native glycolipid is required for optimal binding of antibodies. Analysis of four individual sera (G530, TR752, TR770, and G583) demonstrated the importance of the second sugar, 2,3-di-*O*-methylrhamnose, for optimum binding to anti-PGL-I antibodies, in that ND-O-BSA showed higher, or comparable, reactivity to PGL-I, but D-O-BSA showed negligible or low reactivity. In particular, a dramatic difference in response was seen in specimen G583 giving further support to the evidence for the need for the second and possibly the third sugar for optimal binding. Analysis of the rest of these › specimens also supported the evidence that the entire structure of the terminal region of PGL-I is required for maximum reaction with anti-PGL-I antibodies.

In summary, the study showed that among any large collection of lepromatous leprosy sera, there were some that were reactive to the holistic native antigen, PGL-I, but that did not respond to ND-O-BSA, implying that such antibodies preferentially recognize the terminal 3-*O*-methylrhamnopyranosyl unit of PGL-I or,indeed the phenol group itself. Accordingly, we recently synthesized the entire trisaccharide unit of PGL-I in the form of the *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→39)-oxynonanoyl-BSA (natural trisaccharide-octyl-BSA) (NT-O-BSA) (Figure 2).

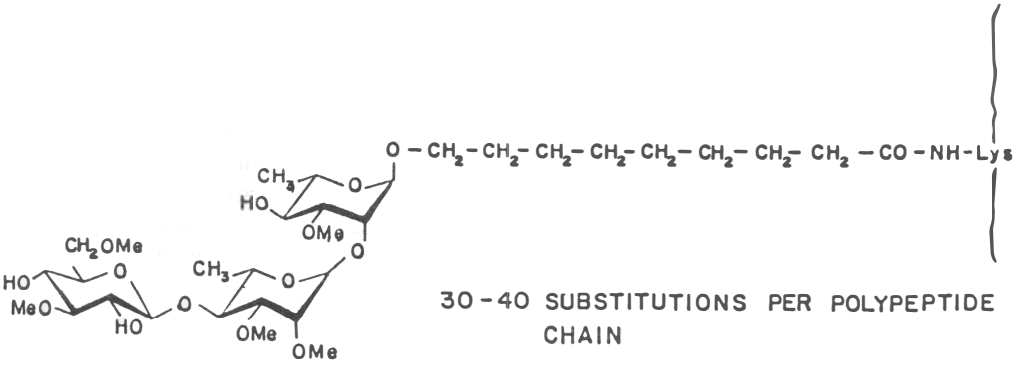


Figure 2. Trisaccharide-containing neoglycoprotein.

The synthetic methods for NT-O-BSA used were essentially those of Wessel and Bundle (27) and Lemieux *et al.* (28), and success of the strategy was dependent on the ability to apply block synthesis. A key intermediate used to establish α-1,2-rhamnopyranoside linkage was 2-*O*-acetyl-4-*O*-allyl-3-*O*-methyl- α-L-rhamnopyranosyl chloride which was obtained in almost quantitative yields from 4-*O*-allyl-3-*O*-methyl-1,2-*O*-(methoxyethylidne)-β-L-rhamnopyranose. An *O*-benzyl group on the 4-0H position was used as a «persistent» blocking agent. Thus, the glycosyl acceptor, 8-methoxycarbonyloctyl 4-*O*-benzyl-3-*O*-methyl-α-L-rhamnopyranoside was condensed with the glycosyl donor in the presence of silver trifluoromethane sulphonate and N,N-tetramethylurea, giving the disaccharide product in high yield (75%) and stereospecificity. Deacylation followed by methylation using methyl trifluoromethanesulphonate gave the 8-methoxycarbonyloctyl 2-*O*-[4′-*O*-allyl-2′,3′-di-*O*-methyl-α-L-rhamnopyranosyl]-4-*O*-benzyl-3-*O*-methyl-α-L-rhamnopyranoside which was subjected to partial deblocking at the 4′ position. Subsequent condensation with a second glycosyl donor, 2,4-di-*O*-acetyl-3,6-di-*O*-methyl-α-D-glucopyranosyl bromide, following Helferich conditions gave the protected trisaccharide which upon deacylation and debenzylation gave the 8-methoxycarbonyloctyl 2-*O*-(4-*O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-2-3-di-*O*-methyl-α-L-rhamnopyranosyl)-3-*O*-methyl-α-L-rhamnopyranoside in overall 63 % yield. The hydrazide was coupled to BSA *via* its intermediate acyl azide; a coupling ratio of 40:1 haptens to protein was obtained. The structure of the final NT-O-BSA is shown in Fig. 2.

In order to examine the efficacy of NT-O-BSA in the serodiagnosis of leprosy, a total of 199 serum specimens were obtained from leprosy patients presenting at the Clinic of the U.S. Public Health Service, Seton Medical Center, Daly City, CA (courtesy of Dr. R.H. Gelber). Patients had been classified clinically and pathologically according to the Ridley and Jopling scale. The ELISA protocol described previously by Cho. *et al.* (15) was employed with minor modifications; the wetting agent, Tween 20, was included in washing solution at a concentration of 0.05% (v/v) only when the neoglycoproteins were being used, i.e., it was not used when PGL-I was the solid phase antigen. The concentration of the individual antigens was standardized by block titration against pooled lepromatous leprosy serum to arrive at an absorbance value (A_{490}) of 1.200.

A comparison 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 different periods showed good correlation between the two antigens in ability to detect anti-glycolipid IgM antibodies. However, the sensitivity rate was appreciably higher when NT-O-BSA was used; 106 patients were seropositive *versus* 97 when PGL-I was used. Perhaps more importantly, NT-O-BSA showed a lesser propensity to react non-specificially with sera from an asymptomatic control population; only three (1.8%) of 169 sera reacted positively to NT-O-BSA compared to seven (4.1%) for PGL-I. Thus, NT-O-BSA showed appreciably greater sensitivity and specificity than the native glycolipid, added to greater felicity in usage due to its water solublility and its amenability to simpler serological tests.

In addition, in parallel studies, Tsuyoshi Fujiwara (Nara University, Nara, Japan) has also synthesized the entire triglycosyl-phenyl unit of PGL-I in the form of a neoglycoprotein, the *O*-(3,6-di-*O*-methyl-β-D-glycopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-α-L-rhamnopyranosyl)-(1→2)-*O*-(3-*O*-methyl-α-L-rhamnopyranosyl)-phenylpropionyl-BSA (popularly called natural trisaccharide-phenylpropionyl-BSA; NT-P-BSA). Previously, he had synthesized the *O*-(3,6-di-*O*-methyl-β-D-glucopyranosyl)-(1→4)-*O*-(2,3-di-*O*-methyl-α-L-rhamnopyranosyl)-phenylpropionyl-BSA; ND-P-BSA (see ref. 29). In extensive interlaboratory studies, near-perfect harmony was observed between all of these neoglycoproteins; correlation coefficients (r) were within the range of 0.884-0.990; agreement was<88%; specificity was in the region of 1.8% (i.e., three positive sera of a control population of 169). In particular, the correspondence between NT-O-BSA and NT-P-BSA was quite extraordinary, a gratifying fact since the products were synthesized by different investigators on different continents.

Recently, Brett *et al.* (30) reported on the synthesis and activities of yet a third generation of PGL-I-related disaccharide-containing neoglycoproteins. The strategy was as described above in that terminal mono- or disaccharides were coupled to BSA by reductive amination; the synthetic protocols were novel and elegant (21). Again, all products arising in this study were highly active in ELISA and showed good concordance with the native PGL-I.

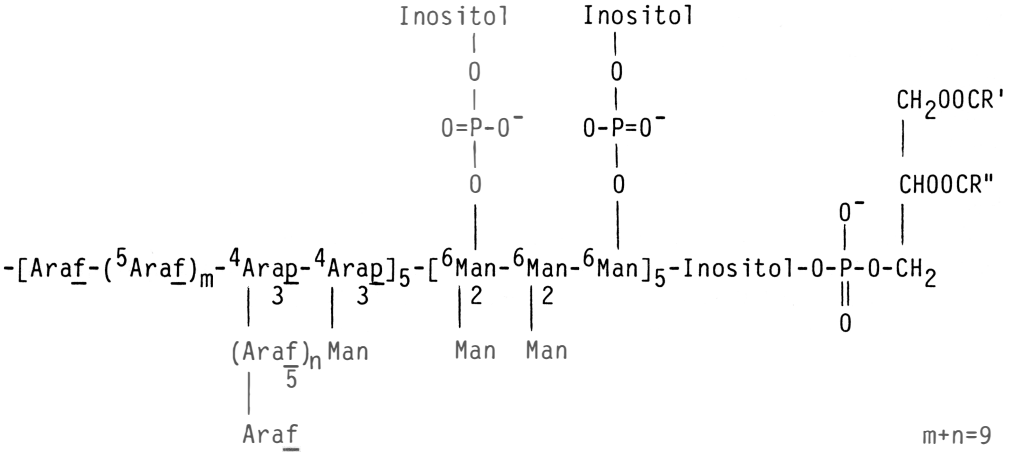
Lipoarabinomannan of *M. leprae;* novel structural and antigenic features

We have previously described how we uncovered and conducted preliminary structural work on one of the dominant antigens of the leprosy bacillus, and arabinose- and mannose-containing phosphorylated lipopolysaccharide, which we called LAM-B [31]. We have since conducted further work on the structure of this extraordinary compound, and the entire story can be summarized briefly as follows.

LAM-B was first recognized as a major soluble carbohydrate-containing polymer in *M. leprae,* which was excluded from gel filtration columns and was highly reactive against lepromatous leprosy sera in ELISA. Purification was accomplished by anion exchange and gel filtration chromatography in detergent; it was recovered in large quantities (15 mg/g bacteria). Thus, it is a major component of the leprosy bacillus. On polyacrylamide gel electrophoresis, it yielded a broad diffuse band, in the vicinity of proteins of 30-35 K M_r, and it appeared homogeneous by polyacrylamide gel electrophoresis and gel filtration. Besides arabinose and mannose, it contained glycerol and phosphate and was acylated by lactate, succinate, palmitate and 10-methyloctadecanoate (tuberculostearate). Permethylation studies showed that the glycerol and, thus, probably, the two long chain fatty acids, exist as a glycosidically linked diacylglycerol. We also made the intriguing observation that the phosphate content of LAM-B existed in two populations. About half of the phosphate was released by relatively mild alkalinolysis. This phosphate was identified by thin layer chromatography, and gas chromatography/mass spectrometry as myoinositol-1-phosphate and must exist in a characteristic alkali-labile phosphodiester format. Recently, we were more surprised to ob-

serve that the second, alkali-stable phosphate population also existed as inositol-1-phosphate; it obviously is in a phosphodiester linkage format different from the alkali-labile inositol-1-phosphate. Recently, we have successfully released the phosphates of this latter population of inositol-1-phosphates by reacting LAM-B with 10 % NH_4OH in a metal «bomb» at 150°C. It now appears that this population of inositol-1-phosphates exists as glycocidically bound phosphatidylinositol mannosides.

The permethylation studies that were initially applied in order to study the comportment of the diacylglycerol moiety were also recently extended to an analysis of the polysaccharide backbone. Terminal mannose (i.e., 2,3,4,6-tetra-*O*-methyl-mannopyranose), terminal arabinose (i.e., 2,3,5-tri-*O*-methyl-arabinofuranose), 5-linked arabinofuranose, 3,4-linked arabinofuranose, 6-linked mannopyranose and 2,6-linked mannopyranose were found in the ratio of 3:1:9:2:1:2, respectively. Thus, one proposed structure of the LAM-B antigen of *M. leprae* is



Based on this structural information, we believe that LAM-B probably traverses the cell wall and periplasmic space of *M. leprae* to be finally anchored in the cytoplasmic membrane.

LAM-B reacts readily with antibodies from lepromatous leprosy patients and monoclonal antibodies in plate and nitrocellulose ELISA and on electrophoretic immunoblots, and is immunologically cross reactive with a like product from *M. tuberculosis* [31]. LAM-B is clearly the pervasive «30-35 Kd glycoprotein» of the leprosy bacillus [32,33] and may be the long-sought lipoteichoic acid-like polymer of *Mycobacterium* spp. with a role in cell wall physiology, macrophage recognition, and, perhaps, involvement in cross-protective immunity.

Given the high immunogenicity of LAM-B, we recently applied it in parallel with PGL-I to the serodiagnosis of leprosy in order to determine whether it was more efficacious than the latter in showing evidence of disease at the paucibacillary end of the leprosy spectrum and, by correlating the levels of anti-PGL-I and anti-LAM-B antibodies, thereby better define the status of incubating leprosy and help enunciate the pathobiology of the disease.

Since some variations on the basic LAM structure are to be expected in all mycobacterial species, including the ubiquitous environmental mycobacteria, it is to be expected that all humans should be exposed and thereby perhaps have natural antibodies to LAM-B. Indeed, among 236 healthy control individuals, 19 (8.1 %) had antibody levels slightly over the criterion for seropositivity (mean absorbance plus three times standard deviation). On the other hand, 43 (81.1 %) of 53 tuberculosis patients showed anti-LAM-B antibodies, and the mean

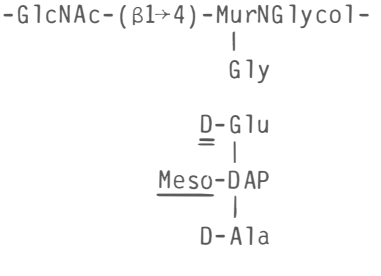
absorbance, A_{490} 1.021, was considerably higher than the A_{490} 0.174 value for the healthy control population. Among 63 tuberculoid leprosy patients, 36 (57.1 %) were seropositive, and 119 (87.5 %) of 136 lepromatous leprosy patients were positive. Thus, in the context of leprosy, a merit of LAM-B lies in its relative effectiveness to detect anti-mycobacterial antibodies in tuberculoid leprosy; the general consensus is that PGL-I has at most a seropositivity rate of about 30 % at this end of the disease spectrum. Indeed, when we compared antibody titres in tuberculoid leprosy patients, the majority (*ca.* 80 %) of the tuberculoid sera were negative to PGL-I, whereas about 60 % were positive to LAM-B and had high-titre antibody levels. Of course, PGL-I and the related neoglycoproteins, unlike LAM-B, show near absolute selectivity for leprosy as opposed to other mycobacterial diseases. Accordingly, LAM-B may be used as a common antigen for screening risk populations to determine the degree of exposure to all mycobacterial species. When necessary, PGL-I or its synthetic analogs may then be used as a selective antigen for incubating lepromatous leprosy.

We have also examined the seropositivity rate and antibody levels to LAM-B in leprosy patients after the implementation of chemotherapy and compared with anti-PGL-I antibodies. A most striking observation was the lack of significant changes in seropositivity rate or mean absorbances among the tuberculoid patients receiving effective chemotherapy. Also, among lepromatous leprosy patients only a moderate decline in seropositivity rate and mean absorbance was evident; even after ten years of successful chemotherapy, the seropositivity rate among the lepromatous leprosy patients was over 85 %. This was the most surprising feature of the study and indicates that LAM-B itself and anti-LAM-B IgG antibodies persist beyond overt evidence of bacillary load and beyond the period when PGL-I itself [34] and its antibodies are cleared. The reason for the survival of antibodies to LAM-B may be that they are of the long-lived IgG class compared to the IgM antibodies evoked by PGL-I. Alternatively, LAM-B, being associated with the internal bacterial envelope, in contrast to extracellular PGL-I, may intercalate into host membrane, thereby continuously evoking humoral immunity.

Thus, antibodies to LAM-B are more prevalent than anti-PGL-I antibodies and persist longer during the course of chemotherapy. Clearly, as a consequence of this phenomenon, anti-PGL-I or PGL-I itself, rather than LAM, provide a more accurate yardstick of successful chemotherapy. The consequence of persisting anti-LAM-B IgG antibodies and perhaps of LAM-B itself in immune complex deposition and reactional states of leprosy is an issue that requires serious investigation.

The cell wall skeleton of *M. leprae*

Draper [35] has indicated that the basic peptidoglycan subunit of the cell wall of *M. leprae* contains features that are both typical of mycobacteria and specific for *M. leprae*. Thus, N-glycolyl muramic acid is present rather than N-acetylmuramic acid. However, unlike all other mycobacteria, the amino acid at the $-NH_2$ terminus of the tetrapeptide side chain is glycine rather than L-alanine. Thus, the basic peptidoglycan subunit of *M. leprae* is



In addition, the nature of the cell wall mycolic acids has been examined in detail [36,37]. However, other than those two moieties, nothing is known of the basic composition of *M. leprae* cell walls or of the basis of its inherent immunogenicity. As a prelude to such an examination, we have recently established that the cell wall associated polysaccharide of *M. leprae* is composed almost solely of arabinose and galactose, and, in this respect, it is like all other mycobacteria. However, the ring form of these sugars and linkage pattern may be distinct. Conventional linkage analyses in which the partially *0*-methylated sugars were examined by gas chromatography-mass spectrometry showed the expected terminal Ara*f* and 2-linked Ara*f* units. However, methylation analysis could not distinguish between 5-linked Ara*f* and 4-linked Ara*p*, 3,5-linked Ara*f* and 3,4-linked Ara*p*, and 5-linked Gal*f* and 4-linked Gal*p*. To resolve these questions, the permethylated cell wall was subjected to partial acid hydrolysis, reduced, ethylated and the products subjected to GC/MS. It was at once obvious that all of the Ara was in the furanose form and, indeed, so also was all of the Gal, and, in fact, no galactopyranose was present in the cell walls of *M. leprae*. We now believe that this is a feature of all mycobacteria rather than *M. leprae* only, and that the literature is incorrect in attributing a →4)-β-D-Gal*p* to the cell wall of mycobacteria; rather, Gal appears consistently as →5)-D-Gal*f*.

We have referred to the cell wall skeleton of *M. leprae* as the bacilli's «last immunogenic frontier». Undoubtedly, as we explore its basic immunochemistry, other novel features will emerge that may be of the essence in explaining the immunological and other reactional responses of the human host to the cell wall of *M. leprae*.

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