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EDITORIALS

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The Phthiocerol-containing Surface Lipids of *Mycobacterium leprae*—a Perspective of Past and Present Work

The recent revelation of the existence in *Mycobacterium leprae* of a number of unique antigenic glycolipids was a landmark event in that it offered for the first time the promise of chemically defined antigen determinants specific to the leprosy bacillus. Others have long pursued this worthy goal but sought proteinaceous or polysaccharide antigens. Recently, Harboe, upon defining the strict criteria for a true species specific antigen, concluded that none had "so far been able to demonstrate any *M. leprae* specific protein antigens"[1].

Immunoreactive glycolipids in *Mycobacterium*

In the context of the *Mycobacterium* genus as a whole the presence of specific glycolipid antigens on the surface of *M. leprae* is not surprising. To date we have described three such groups. All member-serotypes of the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* (MAIS) serocomplex contain type-specific polar "C-mycoside" glycopeptidolipids which are responsible for the Schaefer seroagglutination reactions used to type sub-species of MAIS. Thus, the basic structure shared by all serotypes is a monoglycosylated fatty acyl peptide "core" which is further modified by small variable tri-, tetra- or pentasaccharides which themselves are directly responsible for the serospecificity of individual serotypes[2–4]:

Editor's Note: Due to the necessity for extensive chemical formulas in this text, this Guest Editorial appears in two-column width. Abbreviations: Phe, phenylalanine; aThr, allo*-threonine; Me, O-methyl; Rhap, rhamnopyranose; Fucp, fucopyranose; dTalp, 6-deoxytalopyranose; Glcp, glucopyranose; Phen GL, phenolic diacylphthiocerol glycoside.*

[1] Harboe, M. Significance of antibody studies in leprosy and experimental models of the disease. Int. J. Lepr. **50** (1982) 342–350.

[2] Brennan, P. J. and Goren, M. B. Structural studies on the type-specific antigens and lipids of the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* serocomplex. J. Biol. Chem. **254** (1979) 4205–4211.

[3] Brennan, P. J., Mayer, H., Aspinall, G. O. and Nam Shin, J. E. Structures of the glycopeptidolipid antigens from serovars in the *Mycobacterium avium/Mycobacterium intracellulare/Mycobacterium scrofulaceum* serocomplex. Eur. J. Biochem. **115** (1981) 7–15.

[4] Brennan, P. J., Aspinall, G. O. and Nam Shin, J. E. Structure of the specific oligosaccharides from the glycopeptidolipid antigens of serovars in the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* serocomplex. J. Biol. Chem. **256** (1981) 6817–6822.

Fatty Acyl-CO-NH-D-Phe-D-aThr-D-Ala-L-Alaninol-*O*-(3,4-Me_2-α-L-Rhap)
|
O
|
Oligosaccharide

To date, we have examined the glycopeptidolipid antigens from some 12 serotypes from the MAIS serocomplex and recognized 12 individualistic oligosaccharides[4,5]. Each has been chemically dissected to reveal that even within the realms of a simple tetrasaccharide, there is a region of commonality and a segment of type specificity. Thus, the structure of the tetrasaccharide appendage from serotype 9 is: 2,3-Me_2-α-L-Fucp(1→4)2,3-Me_2-α-L-Fucp(1→3)α-L-Rhap(1→2)6dTalp. The inner reducing-end disaccharide, α-L-Rhap(1→2)6dTalp, is shared by all serotypes whereas the outer nonreducing-end disaccharide is unique to serotype 9. Other examples: in serotype 4 the outer disaccharide is 4-Me-α-L-Rhap(1→2)2-Me-α-L-Fucp(1→4); in serotype 7, it is α-L-Rhap(1→2)α-L-Rhap(1→4); in serotype 8, it is 4,6-(1′-carboxyethylidene)3-Me-β-D-Glcp(1→3); in serovar 25, it is 2-Me-α-L-Fucp(1→4)2-Me-α-L-Fucp(1→3).

Glycolipids of this class also typify the rapidly growing *Mycobacterium chelonei* and *Mycobacterium peregrinum,* and also *Mycobacterium malmoense* and *Mycobacterium simiae* I and II[6]. The characteristic thin-layer chromatography profiles of the pure lipids[7,8] and their specificity and sensitivity in ELISA protocols[9] are now being used to type subspecies of *M. avium, M. intracellulare, M. scrofulaceum* and *M. simiae.* Our first efforts to locate specific immunoreactive lipids in *M. leprae* amounted to a concerted search for glycolipids of this class. Although it came to naught, it did expose the phenolic-phthiocerol glycosides (see below).

A second class of mycobacterial glycolipid antigens of quite extraordinary structure came to light only recently[10]. They are lipooligosaccharides, based on α,α-trehalose [D-glucopyranoside (α1 ↔ 1) D-glucopyranoside], which of itself is an ubiquitous storage disaccharide among mycobacteria, including *M. leprae*[11]. Trehalose, as the dimycocyltrehalose ('cord factor'), is also widespread among mycobacterial species, including *M. leprae*[12]. However, in the trehalose-containing lipooligosaccharides, trehalose is glycosidically linked to a substantial oligosaccharide which in turn is acylated by simple methyl-branched fatty acids[10]. Thus, *M. kansasii* contains seven such lipooligosaccharides of the following composite structure:

Fucp
|
[N-acylaminosugar-Xyl-(Xyl)$_x$]-3-*O*-Me-β-L-Rhap)(1→3)β-D-Glcp(1→3)β-D-Glcp(1→4)α-D-Glc(1 ↔ 1)α-D-Glcp

2,4-dimethyltetradecanoyl or acetyl residues
x = 1–3

The simplest member contains the tetraglucose "core" to which the 3-*O*-Me-Rhap is at-

[5] Brennan, P. J., Aspinall. G. O. and Gray, G. R. (Unpublished observations).

[6] Tsang, A. Y., Barr, V. L., McClatchy, J. K., Goldberg, M., Drupa, I. and Brennan, P. J. The antigenicity of rapidly growing mycobacteria: *Mycobacterium chelonei, Mycobacterium fortuitum, Mycobacterium peregrinum.* (Submitted for publication).

[7] Brennan, P. J., Souhrada, M., Ullom, B., McClatchy, J. K. and Goren, M. B. Identification of atypical mycobacteria by thin-layer chromatography of their surface antigens. J. Clin. Microbiol. **8** (1978) 374–379.

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[9] Yanagihara, D. L., Knisley, C. V., Barr, V. L., Tsang, A., McClatchy, J. K. and Brennan, P. J. ELISA and glycolipid antigens: Application to atypical mycobacteria. (Submitted for publication).

[10] Hunter, S. W., Murphy, R. C., Clay, K., Goren, M. B. and Brennan, P. J. Trehalose-containing lipooligosaccharides: A new class of species-specific antigens from *Mycobacterium.* J. Biol. Chem. (in press).

[11] Elbein, A. D. The metabolism of α,α′-trehalose. Adv. Carbohydr. Res. **30** (1974) 227–256.

[12] Goren, M. B. and Brennan, P. J. Mycobacterial lipids: Chemistry and biologic activities. In: *Tuberculosis.* Youmans, G. P., ed. Philadelphia, Pennsylvania: The W. E. Saunders Co., 1979, pp. 63–193.

tached, and the more polar members are further glycosylated to reach the ultimate complexity in lipooligosaccharide No. 7 which contains all of the above sugars.

Related lipooligosaccharides characterize *Mycobacterium szulgai, M. xenopi, M. gordonae* serotypes and presumably other species. All are endowed with a trehalose-containing tetraglucose core which in turn is modified with sufficient species specific sugars to lend serological specificity to each. All readily react with hyperimmune rabbit antisera in ELISA or by gel diffusion, and thus can be used as a basis for the identification of mycobacteria. A concerted search for glycolipids of this class in *M. leprae* has revealed small quantities of a simple kind, perhaps analogous to that in *M. smegmatis*[13]. In time, we hope to accumulate sufficient material for proper immunochemical characterization.

The presence of a third class of specific glycolipid antigens was first recognized in the course of a deliberate search in *M. leprae* for seroreactive lipid material of the classes just described. This approach led to the recognition of an alkali-stable lipid which yielded distinct lines of precipitation in agar gels against serum from two lepromatous patients and from an infected armadillo[14]. There was no reaction with sera from patients with tuberculosis or a *M. avium* infection or from a normal armadillo. Subsequently, a phenol-phthiocerol glycoside was recognized as the only truly specific lipid in the immunoreactive lipid fractions and, most importantly, large quantities of it and other *M. leprae* specific lipids were isolated from the infected tissue milieu, to the extent of up to 2% dry weight, thereby allowing proper chemical characterization[15, 16]. To date, we have recognized and fully characterized three such phenol-phthiocerol triglycosides[16, 17]:

3,6-Me_2-β-D-Glcp(1→4)2,3-Me_2-α-L-Rhap(1→2)3-Me-α-L-Rhap-α-1→

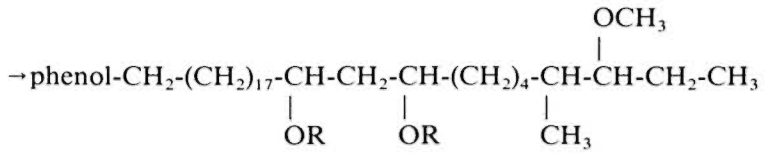
Phen GL-I

3,6-Me_2-β-D-Glcp(1→4)3-Me-α-L-Rhap(1→2)3-Me-α-L-Rhap-α-1→

Phen GL-II

6-Me-β-D-Glcp(1→4)2,3-Me_2-α-L-Rhap(1→2)3-Me-α-L-Rhap-α-1→

Phen GL-III



R = a mixture of three mycocerosic acids; 2,4,6,8-tetramethylhexacosanoate; 2,4,6,8-tetramethyloctacosanoate; 2,4,6,8-tetramethyloctacosanoate.

In addition, the non-phenylated, non-glycosylated dimycocerosylphthiocerol has been isolated in large quantities from infected armadillo tissue and characterized completely[17, 18]:

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[13] Saadat, S. and Ballou, C. E. Pyruvatated glycolipids from *Mycobacterium smegmatis*: structures of two oligosaccharide components. J. Biol. Chem. **258** (1983) 1813–1818.

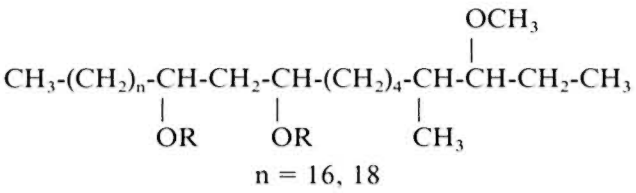
[14] Brennan, P. J. and Barrow, W. W. Evidence for species-specific lipid antigens in *Mycobacterium leprae*. Int. J. Lepr. **48** (1980) 382–387.

[15] Hunter, S. W. and Brennan, P. J. A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J. Bacteriol. **147** (1981) 728–735.

[16] Hunter, S. W., Fujiwara, T. and Brennan, P. J. Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*. J. Biol. Chem. **257** (1982) 15072–15078.

[17] Hunter, S. W. and Brennan, P. J. Further specific phenolic glycolipid antigens and a related diacyl phthiocerol from *Mycobacterium leprae*. J. Biol. Chem. **258** (1983) 7556–7562.

[18] Draper, P., Payne, S. N., Dobson, G. and Minnikin, D. E. Isolation of a characteristic phthiocerol dimycocerosate from *Mycobacterium leprae*. J. Gen. Microbiol. **129** (1983) 859–863.



Young[19] had reported chromatographic evidence for a non-glycosylated phenol-dimycocerosylphthiocerol in *M. leprae* from a human source although as yet we have been unable to confirm this[17]. All of these *M. leprae* specific lipids were found in the *M. leprae*-free supernatant from infected tissue[15–17] which turns out to be a virtual haven of bacillary extracellular products.

Serological activity of the phenolic glycolipids and use in serodiagnosis of leprosy

Since the phthiocerol-containing lipids comprise the bulk of the outer bacillary extensions of *M. leprae*[17], they may represent the primary functional interface between the organism and the host's defense, including antibodies, macrophages, immune lymphocytes, and lysosomal enzymes; thus they afford an unprecedented opportunity to relate *M. leprae* specific entities to the host's immunologic response. Towards this end, we and others have examined the activity of the native phenolic glycolipid against hyperimmune anti-whole *M. leprae* antiserum and serum from patients representing various stages of the clinicohistological spectrum of the disease. Initially, the antigenicity of Phen GL-I had been inferred since it was the only *M. leprae* specific product obtained from the active preparation originally described[14]. However, the untoward lipophilicity of highly purified Phen GL-I, as distinct from the impure preparations, prevented the use of several standard serological assays and, thus, for a while, its antigenicity was a moot question[15]. Payne, *et al.*[20] adopted the ploy of incorporating Phen GL-I into double membrane liposomes[21] which could then diffuse in agarose gels. 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, or 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 anti-glycolipid 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 responded to serum diluted 1/100 from intramuscularly immunized animals[16, 17]. However, these original ELISA conditions were not suitable for the routine diagnosis of human leprosy sera in that sera from healthy humans gave abnormally high absorption values. Recently, we developed a new set of sensitive ELISA conditions that are more in accord with the extreme hydrophobicity of native Phen GL-I and the variable titer of anti-Phen GL-I antibodies in human leprosy sera[22]. 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; the inclusion of heterologous bovine serum albumin and

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[19] Young, D. B. Detection of mycobacterial lipids in skin biopsies from leprosy patients. Int. J. Lepr. **49** (1981) 198–204.

[20] Payne, S. N., Draper, P. and Rees, R. J. W. Serological activity of purified glycolipid from *Mycobacterium leprae*. Int. J. Lepr. **50** (1982) 220–221.

[21] Six, H. L., Young, W. W., Vemura, K-I. and Kinsky, S. C. Efforts of antibody-complement on multiple *vs* single component liposomes. Application of a fluorimetric assay for following changes in liposomal permeability. Biochemistry **13** (1974) 4050–4058.

[22] Cho, S-N., Yanagihara, D. L., Hunter, S. W., Gelber, R. H. and Brennan, P. J. Serological specificity of phenolic glycolipid-I from *Mycobacterium leprae* and use in serodiagnosis of leprosy. Infect. Immun. (in press).

homologous normal goat serum in buffer to prevent nonspecific binding of antibodies to the polystyrene substratum. The sensitivity of the assay is impressive; 2 μg/μl 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 Phen GL-I is primarily immunoglobulin M, to the extent of about 80% of total measured immunoglobulins (IgG, IgM and IgA)[22].

Accordingly IgM response to Phen GL-I was analyzed in sera from over 500 patients attending some of the various clinics of the U.S. Public Health Service system. Not all of the serological data has yet been correlated with the clinical portfolio on these patients, and therefore only a small group of 48 patients attending Dr. Robert Gelber at Daly City, California, will be discussed in the present context.

Twenty-nine of 33 (88%) lepromatous (LL, LI, BL) patients' sera demonstrated antibody levels significantly higher (>3 S.D.) than those seen in a healthy population (N = 23). The mean value for the lepromatous leprosy patients (0.580 ± 0.514) was significantly higher ($p < 0.01$) than for healthy subjects (0.048 ± 0.016). Treatment of lepromatous leprosy for two or more years resulted in significantly lower anti-Phen GL-I IgM values ($p < 0.01$). Of the lepromatous leprosy patients treated for less than two years, 23 of 24 (96%) were positive (0.737 ± 0.519), while for those treated for more than two years, 6 of 9 (67%) were positive (0.162 ± 0.107). However, even in this latter group, the mean value was significantly higher ($p < 0.01$) than in healthy subjects.

Of 13 tuberculoid (TT, BT) patients studied, eight (62%) had significant elevation of IgM antibody to the *M. leprae* specific glycolipid (0.186 ± 0.251); the mean antibody level of tuberculoid patients was significantly higher ($p < 0.05$) than that of healthy controls, and significantly lower ($p < 0.01$) than that of the lepromatous patients. When 5 of the leprosy patients who had initially shown positive sera were re-studied one month to one year later, the titers remained positive, either staying the same (2 patients) or decreasing (3 patients). No patients with tuberculosis (N = 12; mean A_{488} 0.059 ± 0.022) or patients with atypical mycobacterial infections (N = 15; mean 0.038 ± 0.016) demonstrated IgM or IgG seroreactivity to the specific glycolipid.

Of another 113 sera from grouped patients attending the Los Angeles clinic (Dr. Thomas H. Rea) and the New York clinic (Dr. Mark Kaplan; Dr. Marcus Horowitz) 80% in all were judged positive. Of 28 patients classified as tuberculoid, 6 (57%) were positive; of 7 patients classified as borderline, 6 (86%) were positive; and of 78 classified as lepromatous, 68 (87%) were positive, but 100% of these on short-term therapy were positive. Of 24 healthy contacts, only one was judged positive. All of the 300 sera were also analyzed for anti-Phen GL-I IgG and IgA response. Both were present but often at only marginal levels (about one fourth of the IgM response) and showed considerable variation not always reflective of the diseased state.

We have also chemically dissected the Phen GL-I molecule to determine if the trisaccharide appendage is the sole or the dominant antigen determinant. The procedures used, involving alkalinolysis and acid hydrolysis and separation of the various entities by chromatographic means, have been described[16]. In addition, the activities of the triglycosyl diacylphthiocerol from *M. kansasii* (mycoside A)[23] and the monoglycosyl diacylphthiocerol from *M. bovis* (mycoside B)[24, 25] were tested against hyperimmune anti-*M. leprae* rabbit antiserum and pooled sera from lepromatous leprosy patients; none of the latter showed significant activity. In fact, activity was associated only with the moieties containing the trisaccharide entity (i.e., the native or deacylated phenolic glycolipid) indicating that it was truly an *M. leprae* specific antigen determinant.

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[24] Demarteau-Ginsburg, H. and Lederer, E. Sur la structure chimique du mycosides B. Biochim. Biophys. Acta **70** (1963) 442–451.

[25] Knisley, C. V. and Brennan, P. J. Amplification of the structure of the species specific monoglycosyl diacylphenolicphthiocerol from *Mycobacterium bovis*. (In preparation).

Anti-whole *M. leprae* IgG, IgM and IgA have been amply demonstrated in lepromatous leprosy patients, and usually anti-*M. leprae* IgG was about twice as high as IgM[26, 27]. Thus, the anti-*M. leprae* IgM antibodies may be mostly in response to the specific phenolic glycolipids, whereas IgG may be directed primarily against the more common mycobacterial cell wall antigens, such as the arabinogalactan-peptidoglycan complex[28]. Indeed, we have shown that sera from patients with other mycobacterial infections, while devoid of anti-Phen GL-I antibodies, showed high anti-whole *M. leprae* IgG. Anti-*M. leprae* IgM has been attributed to persistent infection[27]. However it is also possible that IgM might be a bystander response to the glycolipid, driven by non-specifically acting lymphokines produced by a specifically activated T cell[29]. Thus, the absence of a direct cell to cell signal delivered by the T cell to the responding B cell could be the reason for the lack of a substantial IgM to IgG switch.

There is now little doubt that the specificity of Phen GL-I, on the one hand, and the sensitivity of ELISA on the other, warrant their use as part of any survey on the incidence of leprosy in a population. Indeed, there is justification for the inclusion of this assay as an integral part of the leprosy vaccine trials now underway.

Recently, we have extended the ELISA strategy to quantitation of the levels of the antigenic glycolipids in body fluids. The approach is simple, involving extraction of plasma, skin biopsy specimens or urine with organic solvents, coating of the polystyrene wells with the total extracted lipid population followed by the application of purified IgG from *M. leprae* immunized rabbits. In an ongoing study we have observed high levels of the phenolic glycolipids in human lepromatous leprosy sera and in lepromas from the same patients. However, considerably more samples of all body fluids must be analyzed before unequivocal statements can be made on the relationship between the clinicohistopathological features of the disease, anti-Phen GL-I IgM and circulating phenolic glycolipids. Whether the phthiocerol-containing lipids are a component of the circulating immune complexes prevalent in patients with untreated leprosy and, thus, possibly involved in leprosy reactions, is not yet known. The next logical step in assessing the role of the glycolipids in the immunological, clinical, and reactional states of the disease is the combined use of the specific antigen and specific monoclonal antibodies to the glycolipid (see below).

Towards a synthetic antigen based on the phenolic glycolipids

To date, we have prepared, in quantity, the following phenolic glycolipids:

3,6-Me_2-β-D-Glcp(1→4)2,3-Me_2-α-L-Rhap(1→2)3-Me-α-L-Rhap-Phen GL
(Phen GL-I from *M. leprae*)[16]

3,6-Me_2-β-D-Glcp(1→4)3-Me-α-L-Rhap(1→2)3-Me-α-L-Rhap-Phen-GL
(Phen GL-II from *M. leprae*)[17,30]

6-Me-β-D-Glcp(1→4)2,3-Me_2-α-L-Rhap(1→2)3-Me-α-L-Rhap-Phen-GL
(Phen GL-III from *M. leprae*)[17]

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[26] Melson, R. and Duncan, M. E. Demonstration of antibodies against *Mycobacterium leprae* both in immunoglobulin G and M in sera from pregnant and non-pregnant lepromatous leprosy patients. Lepr. Rev. **51** (1980) 125–135.

[27] Touw, J., Langendijk, E. M. J., Stoner, G. L. and Belehu, A. Humoral immunity in leprosy: Immunoglobulin G and M antibody response to *Mycobacterium leprae* in relation to various disease patterns. Infect. Immun. **36** (1982) 885–892.

[28] Barksdale, L. and Kim, K. S. Mycobacterium. Bacteriol. Rev. **41** (1977) 217–372.

[29] Enders, R. O., Kushnir, J., Kappler, W., Marrack, P. and Kinsky, S. C. A requirement for nonspecific T cell factors in antibody responses to "T-cell independent" antigens. J. Immunol. **130** (1983) 781–784.

[30] Fujiwara, T., Hunter, S. W., Cho, S-N., Aspinall, G. O. and Brennan, P. J. Synthesis and activity of the di- and trisaccharide determinants of the specific glycolipid antigens from the leprosy bacillus. (Submitted for publication).

2,3-Me_2-α-L-Rhap(1→2)3-Me-α-L-Rhap-Phen GL
(mono-deglycosylated Phen GL-I from *M. leprae*)[16]

3-Me-α-L-Rhap-Phen GL
(di-deglycosylated Phen GL-I from *M. leprae*)[16]

2-Me-α-L-Rhap-Phen GL
("Mycoside B" from *M. bovis*)[24,25]

(2,4-Me_2-Rhap-2-Me-Fucp-2-Me-Rhap)-Phen GL
(Mycoside A from *M. kansasii*)[15,23]

This plethora of phenolic glycolipids, all related in different ways to Phen GL-I from *M. leprae,* presented us with the unique opportunity to probe the precise molecular requirements for binding of Phen GL-I to anti-Phen GL-I IgM from human leprosy sera. Two approaches were used: direct ELISA as described above and ELISA inhibition. Direct ELISA (The Table, compiled from[30]) showed that both of the 3,6-Me_2Glcp-containing glycolipids (Phen GL-I and -II) were about equally active. However Phen GL-III which differed from Phen GL-I in the absence of an *O*-Me substituent on the terminal Glcp unit displayed only about one-third the activity. Likewise, the two partially deglycosylated Phen GL-I showed much less activity, and the monoglycosylated phenolic glycolipid from *M. bovis* and the triglycosylated Phen GL from *M. kansasii* were devoid of activity. Thus, the essential requirement for antigen-antibody interaction is a terminal 3,6-di-*O*-Me-glucopyranosyl unit.

Recently, in conjunction with Tsuyoshi Fujiwara and Gerald O. Aspinall, we undertook chemical synthesis of the trisaccharide determinant of Phen GL-I and the disaccharide and monosaccharide precursors[30]. The products synthesized to date are as follows:

3,6-Me_2-β-D-Glcp(1→4)2,3-Me_2-α-L-Rhap(1→2)3-Me-α-D-Rhap
("natural" trisaccharide from Phen GL-I)

3,6-Me_2-β-D-Glcp(1→4)2,3-Me_2-β-L-Rhap(1→2)3-Me-α-D-Rhap
(same trisaccharide but with the incorrect rha-rha β anomeric link)

3,6-Me_2-β-D-Glcp(1→4)2,3-Me_2-α-L-Rhap
(end disaccharide from Phen GL-I)

6-Me-β-D-Glcp(1→4)2,3-Me_2-α-L-Rhap
(end disaccharide from Phen GL-III)

β-D-Glcp(1→4)2,3-Me_2-α-L-Rhap
(disaccharide devoid of O-CH_3 groups at reducing end Glcp)

These oligosaccharides were inactive in direct ELISA, and consequently an ELISA inhibition assay was developed based on the strategy of quantitating the amount of Phen GL-I required to inhibit Phen GL-I-anti-Phen GL-I IgM binding and relating this to inhibition caused by the synthetic saccharides. The protocol called for direct sonication of the glycolipids in high pH coating buffer and addition to human lepromatous leprosy serum to achieve a dilution of 1:300. In the case of the synthetic saccharides, they were dissolved directly in the buffer prior to adding to human lepromatous leprosy sera. Sera and glycosides were incubated together at 37°C for 3 hr and at 4°C for 16 hr prior to testing activity against Phen GL-I.

The results were quite striking. Phen GL-I drastically inhibited binding: *ca.* 5×10^{-7} M (based on a MW of 2025)[16] resulted in 50% inhibition. As expected, in view of the direct ELISA results, Phen GL-II was about as equally effective. Again in accord with direct ELISA, Phen GL-III was less active; *ca.* 12.5×10^{-7} M was required for 50% inhibition. Partial deglycosylation of Phen GL-I, notably removal of the non-reducing disaccharide, 3,6-Me_2-β-D-Glcp(1→4)2,3-Me_2-α-D-Rhap, abolished most of the antigenic

THE TABLE. *Activity of Phen GL-I, -II, -III, and partially deglycosylated Phen GL-I in binding specific anti-Phen GL-IgM from human lepromatous leprosy sera.*[a]

Glycolipid	Leprosy sera	Normal sera
	(A_{488} ± S.D.)	
Phen GL-I (3,6-Me_2Glcp→2,3-Me_2Rhap→3-MeRhap→Phen GL)	1.520 ± 0.071	0.062 ± 0.011
Mono-deglycosylated Phen GL-I (2,3-Me_2Rhap→3-MeRhap→Phen GL)	0.192 ± 0.037	0.082 ± 0.016
Di-deglycosylated Phen GL-I (3-MeRhap→Phen GL)	0.038 ± 0.010	0.042 ± 0.005
Phen GL-II (3,6-Me_2Glcp→3-MeRhap→3-MeRhap→Phen GL)	1.410 ± 0.075	0.061 ± 0.004
Phen GL-III (6-MeGlcp→2,3-Me_2Rhap→3-MeRhap→Phen GL)	0.590 ± 0.054	0.071 ± 0.003

[a] Polystyrene wells were coated with 50 μl of a sonicate of each glycolipid (5 μg/ml buffer). Human sera were diluted 1:300 with carbonate-bicarbonate buffer. Blocking was conducted with 5% BSA. Goat anti-human IgM peroxidase conjugate was diluted 1:1000 in 20% normal goat serum.

activity. Neither the triglycosyl mycoside A from *M. kansasii* nor the monoglycosyl mycoside B from *M. bovis* caused appreciable inhibition.

Maltose (α-D-Glcp(1→4)α-D-Glcp) and maltotriose (α-D-Glcp(1→4)α-D-Glcp(1→4)α-D-Glcp) were used as controls to study the effects of the synthetic disaccharides and trisaccharides on antigen-antibody binding. Neither they nor the non-methylated Glcp-containing disaccharide, β-D-Glcp(1→4)2,3-Me_2-α-L-Rhap, showed appreciable activity. Only the 3,6-Me_2-β-D-Glcp(1→4)2,3-Me_2-α-L-Rhap disaccharide brought about substantial inhibition of binding of Phen GL-I to anti-Phen GL-I IgM; 1×10^{-6} M caused 50% inhibition. When this disaccharide was replaced in the assay with the "natural" α-linked trisaccharide, there was no enhancement of binding inhibition: 1.1×10^{-6} M caused 50% inhibition. There was no difference in the effects when the β-linked trisaccharide was used. Thus the results of direct serology and binding inhibition with lipids and synthetic saccharides show that the primary site for antibody binding is the non-reducing 3,6-di-*O*-Me-β-D-glucopyranose terminus, with some contribution from the penultimate 2,3-di-*O*-Me-α-L-rhamnopyranose. This information provides us with the essential basis for the synthesis of an artificial antigen, which is now underway.

Recently, we have extended this approach to characterization of some of the monoclonal antibodies directed to the phenolic glycolipids. One such monoclonal antibody, made available by Dr. Barry Bloom, is particularly noteworthy. It is called 46.5 and is an IgG antibody. In direct ELISA, it reacted equally well with Phen GL-I and Phen GL-II. However, it showed little activity against Phen GL-III, thus again indicating the importance of the nonreducing 3,6-di-*O*-Me-β-D-glucopyranosyl terminal in monoclonal IgG-Phen GL-I binding. The mono- and di-deglycosylated Phen GL-I were also virtually inactive. Likewise, in ELISA inhibition assays, only the 3,6-di-*O*-Me-β-D-glucopyranosyl-containing phenolic glycolipids inhibited monoclonal IgG-Phen GL-I binding. Thus, the highly specific monoclonal IgG to Phen GL-I produced by hybridoma technology behaves precisely as the anti-Phen GL-I IgM in sera from human lepromatous leprosy patients. As mentioned above this monoclonal antibody is now being used to assay the levels of Phen GL-I in human leprosy sera.

The phthiocerol-containing lipids and the cell-mediated immune response

One of the most distinctive features of leprosy is the failure of the immune system, a characteristic which distinguishes the disease from other mycobacterial infections. Nowadays, with less emphasis on immunologic deficiencies of human host, attention has turned more to escape mechanisms employed by the parasite[31]. Foremost among these must be the ability to survive in macrophages, even within mononuclear phagocytes activated by

[31] Stoner, G. L. Hypothesis: Do phases of immunosuppression during a *Mycobacterium leprae* infection determine the leprosy spectrum? Lepr. Rev. **52** (1981) 1–10.

sensitized T cells to destroy intracellular parasites. *M. leprae* has a complex life cycle within the phagocytic cell, only part of which is spent within the phagosome system[32]. Thus, *M. leprae* may possess the capabilities to block or delay phagosome-lysosome fusion, in addition to protecting itself from lysosomal degradation once fusion takes place. Imaeda, *et al.*[33], Draper[34], Fukunishi, *et al.*[35], and others have contributed to a firm impression of the capsular ultrastructure of both *M. leprae* and *M. lepraemurium* within phagocytic cells. It is now clear that they are vastly different, thus casting further doubt on murine leprosy as a relevant model for the human disease.

Negative staining and freeze fracture techniques have shown *M. lepraemurium in situ* associated with fibrillar or crystalline extracellular material which apparently is shed into the phagocytic cell. Draper and Rees[36] physically isolated this zonal material from *in vivo* cultured *M. lepraemurium* and upon hydrolysis it yielded those key amino acids and 6-deoxyhexoses known to be associated with "C-mycosides" (see above). Similar material may also be found in media in which *M. avium*[37] or other members of the MAIS complex[38] had been cultured and, indeed, thorough analysis of such material from the Arnold serotype (serovar 20) of the MAIS complex revealed that the bulk of it was composed of the polar C-mycoside glycopeptidolipids containing the specific tetrasaccharide determinant associated with that particular serotype[38]. Draper and Rees[36] first suggested that the C-mycoside capsular material may protect the bacterium and thereby facilitate its parasitic role. Indeed, the primary structure of the glycolipid is suited to this task with a preponderance of unreactive D-amino acids and heavily *O*-methylated 6-deoxyhexoses. The fact that the C-mycosides are phage receptors in some mycobacteria[39] and that the polar versions are the typing antigens of many species and subspecies also point to an external role.

The evidence that the capsular material of *M. leprae* is composed in large measure of the phthiocerol-containing lipids is mostly indirect. Fukunishi and colleagues[35, 40], using freeze etching techniques, observed "small spherical droplets" within the phagolysosomes of human lepromas, and macrophages of nude mice, and *M. leprae*-infected armadillos. These structures were markedly different from the fibrillar material surrounding *M. lepraemurium*. Apparently they are unique to *M. leprae*-infected tissue and are probably synonymous with material described in the older literature as "peribacillary substance," "foamy structure" or "capsular materials." Previously, Hanks[41], largely on the basis that 5%–10% chloroform in aqueous media declumped and dispersed *M. leprae*, had concluded that mycobacterial lipids were the major bonding substances in the electron transparent material. Since the quantities of phthiocerol-containing lipids in infected tissue is in excess of that to be expected from the bacillary load, we have reasoned that they are the likely

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candidates for the extracellular "spherical droplets." According to recent data, 3.7×10^{10} *M. leprae* bacilli, which amounts to 1.443 mg dry weight, produces 1.63 mg of extracellular phthiocerol-containing lipid[17]. The inference is that *M. leprae* excretes a surfeit of the chemically inert phthiocerol-containing lipids which are highly suited to the role of passive protectors of resident *M. leprae* within the phagosome. Direct evidence for a superficial location for the phenolic glycolipids was recently provided by Barrow, *et al.*[42] who produced antibodies to Phen GL-I by injecting rabbits with a complex composed of Phen GL-I-methylated bovine serum albumin[43]. An indirect immunofluorescent antibody procedure showed the preferential location of Phen GL-I on the surface of *M. leprae* and in the surrounding environ after phagocytosis of *M. leprae* by mouse peritoneal macrophages.

Besides being a passive inert defender of *M. leprae*, the phthiocerol-containing lipids may have other key roles to play in facilitating the survival of the bacillus. The impact of mycobacterial antigens with sensitized T cells with the evocation of various lymphokines, and activation of macrophages is considered to be the primary means of defense against mycobacterial infection[44]. However, recent substantial evidence indicates that the depressed cellular immunity observed in mycobacterial diseases is in some instances due to overactivity of suppressor cell systems. Thus, Mehra, *et al.*[45] have shown lepromin-induced suppression of Con A stimulation in most patients with leprosy and have indicated that adherent cells and T_γ cells are involved in this suppressor activity. Since, at least in the case of lepromatous leprosy, cellular unresponsiveness is associated with lesions containing large loads of bacilli, there is the possibility that bacterial products manipulate the immune response in such a manner as to evoke suppressor mechanisms that might protect the bacteria from the immune response. Perhaps, the massive load of phthiocerol glycosides and aglycones are directly responsible for suppression. Indeed, Wadee, *et al.*[46] have shown that mycobacteria delipidated by repeated extraction with diethyl ether failed to suppress lymphocyte blastogenesis. However when the extracted lipid was dispensed by sonication and added to lymphocyte cultures, suppression was observed. Their overall study indicated that lipid material is responsible for the production of a suppressor factor by adherent cells and the suppressor factor will activate T_γ suppressor cells that have the ability to inhibit the blastogenic ability of normal lymphocytes activated by mitogens.

Thus, the phthiocerol-containing lipids in their sheer mass, in their peculiar structural and metabolic inertness, and in the presence on the glycosylated types of a unique antigen determinant, may be key modulators of the immune system in the progression of leprosy. At the very least they provide us with an unprecedented and unequivocal taxonomic marker of the leprosy bacillus.

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