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Analysis of the major antigenic determinants of the characteristic phenolic glycolipid from *Mycobacterium leprae*

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SUMMARY

Antibodies to the major phenolic glycolipid purified from *Mycobacterium leprae* have been demonstrated previously in sera of leprosy but not tuberculosis patients using an ELISA. The major antigenic determinants on this molecule were investigated using antisera raised in rabbits to the purified glycolipid and with a pool of sera from human lepromatous leprosy patients. A small, but significant cross-reaction was observed with the glycolipids from *M. bovis* and *M. kansasii*, which contain the phenolphthiocerol dimycocerosate part of the molecule but have different sugars, and also with a semi-synthetic 'attenuation indicator lipid' which shares the phenolphthiocerol but has no sugars. There was however no cross-reaction with phthiocerol dimycocerosate. The disaccharide, corresponding to the two terminal sugars of the *M. leprae* glycolipid has been chemically synthesized and shown to inhibit the reaction between glycolipid and antibody in the ELISA. The cross-reactivity observed with the *M. bovis* and *M. kansasii* glycolipids was not inhibited by the synthetic disaccharide. These findings suggest that the cross-reactivity is associated with the phenol ring and implies the disaccharide may be a unique antigenic determinant of *M. leprae*.

Keywords *Mycobacterium leprae* ELISA phenolic glycolipid specific disaccharide

INTRODUCTION

The antigenic analysis of *Mycobacterium leprae* has revealed extensive serological cross-reactivity with other mycobacterial species (Gillis *et al.*, 1981, Closs, Mshana & Harboe, 1979). The characterization of species specific antigens for *M. leprae* would therefore be extremely useful for differentiating the disease from tuberculosis and infections caused by 'opportunistic' mycobacteria, and also for diagnosis of subclinical infection. Most investigators have concentrated on protein antigens and the development of monoclonal antibody (MoAb) production has greatly aided these studies. Recently two groups have obtained MoAbs which appear to be specific for protein antigens of *M. leprae* (Gillis & Buchanan, 1982, Ivanyi *et al.*, 1983). In the 'atypical', non-tuberculosis mycobacteria, complex wall or capsular lipids occur which show species specific antigenicity (Brennan, 1981). The chemical characterization of a phenolic glycolipid from *M. leprae* which occurs in large amounts in the isolated bacillus (2% dry weight) and in the surrounding host tissue (2·2 mg/g) therefore raised the possibility that this was a species specific antigen (Hunter & Brennan, 1981). This would be extremely useful as it is a readily available 'by-product' of the leprosy vaccine

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preparation (Draper, 1979). Chemically the glycolipid has a long phenol–glycol skeleton, esterified by long, branched chain fatty acids, to which a trisaccharide is linked. Although a similar phenol–glycol skeleton occurs in several other mycobacterial lipids and glycolipids, which include mycoside A from *M. kansasii* (Gastambide-Odier & Sarda, 1970), mycoside B from *M. bovis* (Demarteau-Ginsburg & Lederer, 1963) mycoside G from *M. marinum* (Sarda & Gastambide-Odier, 1967) and attenuation indicator lipid from some strains of *M. tuberculosis* (Goren, Brokl & Schaefer, 1974), the trisaccharide moiety of the *M. leprae* glycolipid is apparently unique.

The glycolipid was found to be serologically active if the antigenic sites were adequately exposed in aqueous media; either by incorporating it into liposomes in an immunodiffusion test (Payne, Draper & Rees, 1982) or using sodium deoxycholate in the coating solution in an enzyme linked immunosorbent assay (ELISA) (Brett *et al.*, 1983). An ELISA for the glycolipid has also been described by Hunter, Fujiwara & Brennan (1982) and Young & Buchanan, (1983). Patients with various clinical types of leprosy were found to have anti-glycolipid antibodies in their serum. Highest levels were found in lepromatous leprosy patients, while approximately 50% of tuberculoid leprosy patients had detectable anti-glycolipid antibodies (Brett *et al.*, 1983). The latter study also showed that patients suffering from diseases caused by other types of mycobacteria (including tuberculosis) did not have significantly higher levels of anti-glycolipid antibody than normal healthy control subjects.

The present study is concerned with dissecting out the precise molecular epitopes involved in the binding of the *M. leprae* glycolipid to rabbit sera raised against the purified glycolipid or to human lepromatous leprosy sera. This was made possible by the availability of several closely related molecules which differed from the *M. leprae* glycolipid in the number and type of carbohydrate residues and the presence or absence of a phenolic ring (Fig. 1). Further analysis was carried out using the synthetic terminal disaccharide (Gigg, Payne & Conant, 1983) of the *M. leprae* glycolipid.

MATERIALS AND METHODS

Sera. Immune sera were raised against the purified *M. leprae* glycolipid using the method of Alving, Fowble & Joseph (1974). Each rabbit was immunized subcutaneously with 1 ml emulsion consisting of 7 mg glycolipid; 2·5 mg bovine serum albumin in 0·5 ml NaCl (0·154 M) and 0·5 ml Freund's incomplete adjuvant. Animals were boosted 2–3 weeks later with half the above dose, and blood was taken after a further 2 weeks. Sera from 12 patients with untreated lepromatous leprosy (LL), clinically and histologically classified according to the Ridley–Jopling scale (Ridley & Jopling, 1966; Ridley & Waters, 1969) were used. Pooled sera from healthy UK subjects were used as a negative control.

Purification of mycobacterial glycolipids. The *M. leprae* glycolipid was purified from supernatants generated after homogenization of livers and spleens of *M. leprae* infected nine banded armadillos (Draper, 1979). Details of purification by column and thin layer chromatography have

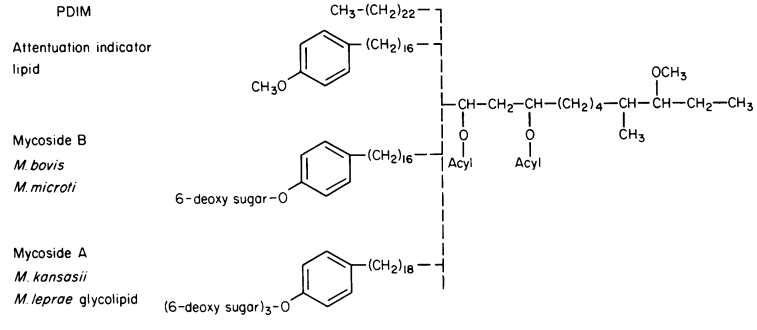

Fig. 1. Chemical structure of the lipids and phenolic glycolipids purified from various mycobacterial species. The acyl groups are palmitoyl or mycocerosyl.

been previously described (Payne *et al.*, 1982; Hunter & Brennan, 1981), as has the structural characterization of the trisaccharide (Hunter *et al.*, 1982). Purified mycoside A from *M. kansasii* and mycoside B from *M. bovis* were kindly donated by S. Hunter & P. Brennan and phthiocerol dimycocerosate of *M. leprae* (PDIM) by D. Minnikin.

Phenolphthiocerol dimycocerosate (ϕPDIM) and attenuation indicator lipid (AIL) were prepared by the method of D. Minnikin (personal communication). ϕPDIM was obtained by methanolysis of purified glycolipid. Glycolipid (9 mg) was dissolved in 50 μl toluene. Methanol (400 μl) was added, followed by 16 μl acetyl chloride. The reaction mixture was heated at 60°C overnight in a sealed vial. ϕPDIM was extracted from the reaction mixture with 3 ml petroleum spirit (b.p. 40–60°C) and purified by thin layer chromatography on silica gel, using chloroform–methanol (95:5 vol./vol.) as solvent.

AIL, which is the methyl ether of ϕPDIM was prepared from ϕPDIM. Half the ϕPDIM was dissolved in 1 ml dichloromethane. Phase transfer solution (1 ml of aqueous 0·2 M NaOH, 0·1 M tetrabutylammonium sulphate) was added, then 25 μl iodomethane. After mixing for 30 min the layers were allowed to settle. AIL contained in the lower layer was purified by thin layer chromatography on silica gel with petroleum spirit (b.p. 40–60°C) acetone (95:5 vol./vol.) as solvent.

The de-acylated *M. leprae* glycolipid was prepared by reductive deacylation with lithium–aluminium hydride using standard procedures.

Details of the chemical synthesis of the terminal disaccharide, 3,6,di-*O*-methyl-β-D-glucopyranosyl-(1→4)2,3,-di-*O*-methyl-L-rhamnose and its α-propyl glycoside have been described elsewhere (Gigg *et al.*, 1983).

ELISA. Glycolipid preparations were added to polyvinyl chloride microtitre plates (Dynatech Laboratories Inc) at a concentration of 4 μg/ml and incubated for 18 h at 37°C. Several methods were used for suspending the glycolipids in aqueous solution for coating the plates.

(a) Suspension of the glycolipid in sodium deoxycholate 1 mg/ml in 0·01 M phosphate-buffered saline (PBS). This method was used for most experiments including those comparing other mycobacterial glycolipids and lipids with the *M. leprae* glycolipid.

(b)Sonication of the deacylated glycolipid in PBS. Deacylation removes approximately one half of the total lipid from the glycolipid, thereby making it more soluble in aqueous suspensions. This method was suggested by Young & Buchanan (1983).

(c) The intact glycolipid did coat the plates successfully, if first thoroughly sonicated for 10 min in an alkali buffer (carbonate–bicarbonate buffer, 0·05 M, pH 9·6). This buffer is commonly used for coating proteins on to microtitre plates (Hudson & Hay, 1980).

Immobilization of whole mycobacteria onto the wells was carried out as described by Touw *et al.*, (1982). Mycobacterial suspension of approximately 4 μg/ml were added to the microtitre plates (100 μl/well).

The rest of the assay was performed as described previously by Brett *et al.* (1983). The conjugates used were swine anti-rabbit Ig/horseradish peroxidase (1:2,000) and goat anti-human IgM (1:1000) (DAKO products). All assays were performed in triplicate.

In experiments involving the synthetic oligosaccharides an ELISA inhibition assay was developed as these sugars were inactive in the direct assay. The sugars were dissolved directly in incubation buffer (PBS, 0·05% wt/vol. Tween 20). Intact glycolipids were also used in inhibition experiments and these were sonicated in the carbonate–bicarbonate buffer. Sera and sugars-glycolipids were incubated together at 4°C overnight prior to testing their activity in the ELISA.

RESULTS

Comparison of coating procedures

The insolubility of the intact glycolipid in aqueous solution made it necessary to use special methods to attach it to microtitre plates, such that the antigenic sites were exposed. All the methods seemed to be efficient in coating the plates as they had similar activity when tested with the rabbit anti-glycolipid antiserum (Fig. 2).

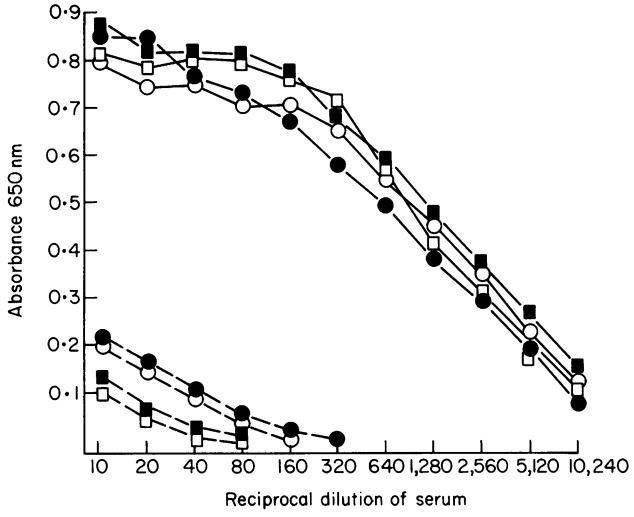

Fig. 2. Rabbit Ig anti-*M. leprae* glycolipid antibody activity (—) against *M. leprae* glycolipid in sodium deoxycholate (●), *M. leprae* glycolipid sonicated in high pH buffer (□), deacylated *M. leprae* glycolipid (■) and whole irradiated *M. leprae* (○). The concentration of coating antigen was 4 μg/ml. Binding of these antigens to normal rabbit serum (– – –).

The rabbit anti-glycolipid serum was also found to bind to whole irradiated *M. leprae* (2·5 megarad) in solid phase ELISA and showed similar activity to that of the purified glycolipid.

Fig. 3 shows the effect of varying the concentration of the glycolipid (with deoxycholate) used to coat the plates. The assay was found to be very sensitive, detecting less than 10 ng/ml of glycolipid.

Cross-reactivity of M. leprae *glycolipid with related molecules*

The cross-reactivity of several lipids and glycolipids purified from other mycobacteria with the *M. leprae* glycolipid was assessed using a rabbit antiserum raised against the purified *M. leprae* glycolipid and also a pool of human LL sera (Fig. 4). No cross-reactivity was detected to PDIM which shares most of the lipid backbone with the *M. leprae* glycolipid but has no phenol group or carbohydrate. Substantial cross-reactivity, to a similar extent, was found with AIL, φPDIM and the *M. kansasii* and *M. bovis* glycolipids although antigen–antibody binding was less than to the *M. leprae* glycolipid. These molecules all share most of the lipid backbone and phenol ring, but differ in their carbohydrate residues which may or may not be present (Fig. 1). The degree of cross-reaction

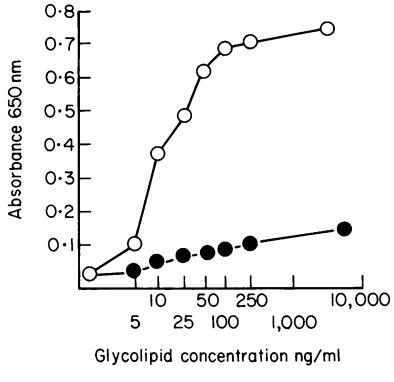

Fig. 3. Effect of decreasing *M. leprae* glycolipid concentration used for coating microtitre plates on the binding of rabbit Ig anti-glycolipid antiserum at 1/10 dilution (○) and of normal rabbit serum (●).

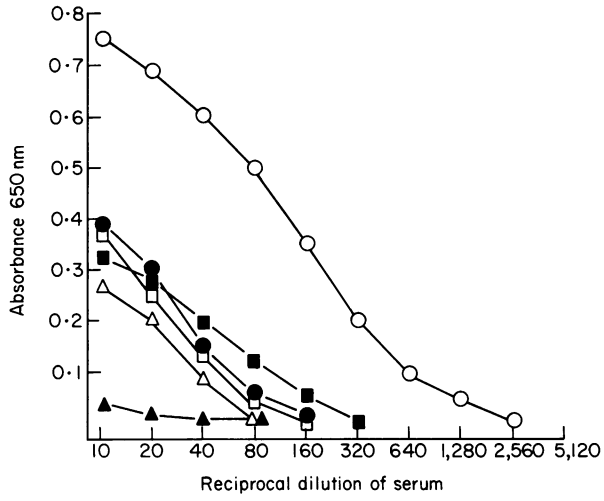

Fig. 4. Rabbit Ig anti-*M. leprae* glycolipid antibody activity against the *M. leprae* glycolipid (○); the *M. kansasii* (●) and *M. bovis* (□) glycolipids, ϕPDIM (■), AIL (△) and PDIM (▲). The absorbances obtained with normal rabbit serum were subtracted from the immune serum readings.

was similar with all these molecules being about 35% of the *M. leprae* glycolipid value at 1/20 serum dilution, with the cross-reactivity being diluted out at dilutions > 1/100. Similar results were obtained with sera from a pool of LL patients.

Inhibition of antigen–antibody binding in the ELISA

Almost total inhibition of antigen–antibody binding, in the ELISA, occurred when the *M. leprae* glycolipid (at concentrations > 10 μg/ml) was co-incubated with the rabbit anti-glycolipid immune serum (Fig. 5a). The inhibitory effects of the synthetic sugars on antigen–antibody binding in the ELISA were also investigated. The synthetic disaccharide corresponding to the terminal 2 sugars on the *M. leprae* glycolipid (3,6-di-*O*-Me-D-glucose linked β-1→4 to propyl 2,3,di-*O*-Me-L-rhamnose) resulted in substantial inhibition of binding of the *M. leprae* glycolipid to the anti-glycolipid antiserum (Fig. 5a). The response was however not inhibited more than 60% even at disaccharide concentrations of 1 mg/ml. No inhibition was seen when the three single sugars were added separately or together; the results with 3,6-di-*O*-Me-D-glucose are shown in Fig. 5a, but the other two sugars gave essentially the same result. Sucrose and maltose were used as control unrelated disaccharides and these caused no inhibition of antigen–antibody binding.

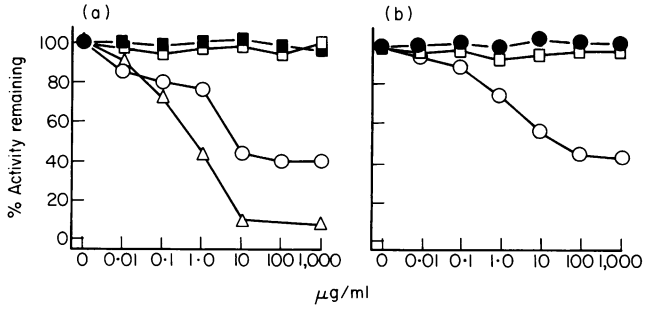

Fig. 5. Activity in ELISA inhibition of the *M. leprae* glycolipid and synthetic oligosaccharides against rabbit Ig anti-glycolipid serum. (a) *M. leprae* glycolipid was used to coat microtitre plates and immune serum incubated with varying concentrations of sonicated glycolipid (△); disaccharide (○); 3,6 di-*O*-methyl-D-glucose (□) and maltose (■); (b) *M. leprae* (○), *M. kansasii* (●) and *M. bovis* (□) glycolipids were used to coat microtitre plates. The anti-*M. leprae* glycolipid serum was pre-incubated with the synthetic disaccharide.

Fig. 5b shows that although substantial inhibition of binding of the *M. leprae* glycolipid to anti *M. leprae* glycolipid antiserum occurs with synthetic disaccharide, no such inhibition is seen when the *M. kansasii* and *M. bovis* glycolipids were used as the coating antigens.

The ability of the disaccharide to inhibit antigen–antibody binding by the sera of 12 untreated lepromatous leprosy cases was investigated (Table 1). All except two of these sera were inhibited to some degree by the disaccharide, but there was considerable variation between individuals.

DISCUSSION

The evidence available from this study strongly suggests that the unique disaccharide on the *M. leprae* phenolic glycolipid is an immunologically specific determinant of *M. leprae*. However it does appear that the glycolipid molecule as a whole is slightly cross-reactive with related molecules from other mycobacteria. This cross-reactivity seems to be associated with the phenolic group which all these compounds have in common. Analysis of the immunological relationship between these lipids and glycolipids was aided by the production of antiserum raised in rabbits specifically against the purified *M. leprae* glycolipid. A high titre pool of lepromatous patients sera, however, gave similar results. These sera showed a small but significant cross-reactivity with the glycolipids from *M. bovis* and *M. kansasii* which contain the phenolphthiocerol part of the molecule but different sugars. There was no detectable cross-reaction with PDIM. Also, similar titration curves were found with the native and deacylated *M. leprae* glycolipid (Fig. 1), indicating removal of the fatty acid chains did not decrease the antigenicity of the molecule. The latter two observations therefore suggest that the anti-glycolipid antibody response is not directed against determinants on the lipid part of the molecule and indicates that the phenol ring may be the cross-reactive determinant. Confirmation of this was obtained by deglycosylation to give ϕPDIM and subsequent methylation to form a 'semi-synthetic' AIL. Both ϕPDIM and AIL showed the cross-reaction. These results differ from those reported in a recently published abstract (Yanagihara *et al.*, 1983), in which antisera against the *M. leprae* glycolipid did not cross-react with PDIM, the diacylphthiocerol, nor with the phenolic

Table 1. Inhibitory effects of disaccharide* on binding of lepromatous leprosy patients sera to the *M. leprae* glycolipid

Patient	% Inhibition
LL 1	57
2	37
3	55
5	65
10	20
11	49
12	10NS†
14	5NS†
15	23
16	22
17	57
18	22

* Disaccharide concentration was 500 μg/ml.

† No significant inhibition was found using Student's *t*-test. All other results showed significant inhibition ($P < 0{\cdot}05$).

glycolipids with different sugar composition from other mycobacteria. Some cross-reactivity with the *M. kansasii* glycolipid, however has recently been reported by Young & Buchanan (1983). The reason for this discrepancy is unknown at the present time.

Further exploration of the nature of the antigenic determinants on the *M. leprae* glycolipid was carried out using the synthetic disaccharide from this molecule. The synthetic disaccharide (3,6-di-*O*-Me-β-D-glucose (1→4)propyl2,3-di-*O*-Me-L-rhamnose) corresponding to the two terminal sugars of the glycolipid, partially inhibited the reaction between the glycolipid and the antibody. No inhibition was seen with any of the single sugars added either separately or together.

It was also found that the cross-reactivity observed with either the *M. kansasii* or *M. bovis* glycolipids was not inhibited by the synthetic disaccharide. This confirms the view that the cross-reactivity observed in the rabbit serum is associated with the phenol ring and implies that the disaccharide is an unique antigenic determinant of *M. leprae*.

Synthesis of the trisaccharide from the *M. leprae* glycolipid is in progress and it is hoped to conjugate this to a protein carrier to produce a synthetic antigen in a form easier to handle than the native glycolipid. As show in Fig. 2, use of detergents, removal of fatty acid chains or very thorough sonication in high pH coating buffer are required to ensure that the antigenic determinants are adequately exposed in the aqueous environment of the ELISA. Other workers have also found the deacylated glycolipid to be excellent for coating the plates (Young & Buchanan, 1983) and it is certainly advantageous not to have to use a detergent. As sonication of the intact glycolipid in high pH buffer also gives good coating this may be the simplest method for large scale epidemiological surveys, as it does not require detergents or any further chemical treatments; at least until the synthetic trisaccharide antigen is available in large quantities.

It was reassuring to find that the rabbit anti-glycolipid antibody bound to whole *M. leprae*, indicating the oligosaccharide part of the glycolipid is exposed on the bacterial surface. This activity could be almost totally inhibited by absorbing the antisera with whole bacilli or sonicated *M. leprae* glycolipid.

The finding that antigen–antibody binding of sera from all but two lepromatous patients was inhibited by the synthetic disaccharide indicates that the majority of individuals do make antibodies to the disaccharide. The variation in the amount of inhibition between individuals could be a consequence of the polyclonal nature of the antibody response or the avidity/affinity and titre of the antibody response. These possibilities are currently under investigation.

The ELISA appears to be an extremely sensitive assay detecting < 10 ng/ml (1 ng/well) glycolipid. Reverse assays using specific anti-glycolipid antibodies to detect low levels of glycolipid in serum or tissues may have use for diagnosis of subclinical infection and following degradation of the bacilli following chemotherapy.

The supernatants used to prepare the lipid were 'by-products' formed in the production of purified *M. leprae* for the UNDP/World Bank/WHO IMMLEP programme. We would like to thank Dr A. Andreoli (Dhoolpet Leprosy Research Centre, Hyderabad) for the leprosy sera, Dr D.E. Minnikin (Newcastle University) for PDIM from *M. leprae* and methods to prepare φPDIM and AIL, Dr S. Hunter and Dr P.J. Brennan (Colorado State University) for mycoside A from *M. kansasii* and mycoside B from *M. bovis*. We are grateful to Dr M.J. Colston for his critical evaluation of the manuscript and also to Mr R. Butler for excellent technical help.

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