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Specific binding of phenolic glycolipid antigens from *Mycobacterium bovis* BCG with antibodies

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We studied the molecular binding specificity of two rabbit polyclonal sera generated against phenolic glycolipid antigens namely PheG1 B and PheG1 B-3 from *Mycobacterium bovis* BCG. PheG1 B is the well-known mycoside B (2-*O*-Me-α-L-Rha*p* 1→aglycone), while PheG1 B-3 is a recently found glycolipid (α-L-Rha*p*-(1→3)-2-*O*-Me-α-L-Rha*p* 1→ aglycone). The interaction specificity was mainly explained in terms of the cavity volume of the antibodies paratope. The anti-PheG1 B antibodies paratope fits the 2-*O*-Me-α-L-Rha*p* ligand, while that of anti-PheG1 B-3 binds the disaccharide moiety of PheG1 B-3, and, with a higher affinity, the monosaccharidic unit localized at the non-reducing end. The B-3 antigen affinity is higher than that of antigen B for their homologous antibodies. This can be explained by the fact that the antibodies against phenolic glycolipid B-3 bind optimally to two sequential glycosyl residues suggesting the presence of two subsites. The immunoglobulin subsite with the major affinity binds the monosaccharidic unit localized at the non-reducing end.

Mycobacterium bovis BCG; Epitope; Phenolic glycolipid

1. INTRODUCTION

In the last decade it has been shown that most myocardial cell walls contain species-specific immunoreactive glycolipids [1–3]. These glycolipids are subdivided according to their structure into four groups; the phenolic glycolipids (PheG1), the trehalose-containing lipooligosaccharides (LOS), the C-mycoside glycopeptidolipids (GPL) and finally the serine-containing glycopeptidolipid [4].

Whatever the structure of these antigens their epitope was delineated in the sugar part. More precisely their species specificity arises from the unique structure of the monosaccharide unit located at the non-reducing end of the oligosaccharide part. In some cases, the terminal monosaccharide structure was assigned to monosaccharides newly-found in nature, such as, 2,6-dideoxy-4-*O*-Me-*arabino*-α-D-hezopyranoside [5,6], 4,6-dideoxy-2-*O*-Me-3-C-Me-4-(2′-methoxypropionamido)-α-L-*manno*-hexopyranoside [7,8], and 4-lactylamido-3-*O*-Me-4,6-dideoxy-β-Glc*p* [9] which comprised the *M. kansasii* phenolic glycolipid, the *M. kansasii* LOS and *M. avium* complex serovar 12 GPL, respectively. In other cases, the distal monosaccharide structure arises from ubiquitous monosaccharides such as α-L-Rha*p*, α-L-Fuc*p*, β-D-Glc*p*, and α-D-Man*p* but bearing either methoxyl or acetoxyl groups [10–14].

From a structural point of view, the process of carbohydrate epitope binding by antibodies is a key factor in the understanding of the mechanisms involved in the specific recognition of a carbohydrate ligand by immunoglobulins [15]. Recently Nashed et al. [16] showed that IgA monoclonal anti-isomaltose antibody binds optimally to four sequential glucopyranosyl residues and that the protein subsite possessing the major affinity binds to the terminal non-reducing glucosyl unit.

Our laboratory is involved in the purification and structural elucidation of mycobacterial immunoreactive glycolipids. We have recently described the presence, in the cell walls of *Mycobacterium bovis* BCG, besides the well known mycoside B (2-*O*-Me-α-L-Rha*p* 1→diacylphenolphthiocerol), of two newly found phenolic glycolipids, namely PheG1 B-2 and PheG1 B-3. Their carbohydrate structures were assigned respectively, to α-L-Rha*p* and α-L-Rha*p*-(1→3)-2-*O*-Me-α-L-Rhap [17]. In the present work the ligand binding specificity of rabbit polyclonal anti-PheG1 B and anti-PheG1 B-3 sera are determined. The experimental approach was based on affinity measurements by ELISA of structurally related phenolic glycolipids from other mycobacterial species and synthetic methyl glycosides. The process of carbohydrate epitope binding as well as the recognition mechanisms will be discussed according to the immunoglobulin cavity size and hydrogen bonding interactions.

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2. MATERIALS AND METHODS

2.1. *Isolation of phenolic glycolipids*

The purification and characterization of glycolipids from *M. bovis* BCG and *M. kansasii* have been described in previous work [6,17]. Purification of PheG1 M from M. marinum was performed as described by Fournié et al. [6].

2.2. *Monosaccharides*

Methyl glycosides were purchased from Sigma except methyl-α- and β-L-rhamnopyranoside, which were obtained after treatment of α-L-rhamnopyranose by anhydrous methanol/2 M HCl, for 2 h at 80°C and purification by HPLC as previously described [18]. Methylated methyl α-L-rhamnopyranosides were synthesized and purified by HPLC as previously described [18].

2.3. *Antiserum preparation*

Two 2-month-old 'fauve de Bourgogne' rabbits were inoculated intradermally with 3 mg of phenolic glycolipid B or B-3 in incomplete Freund's adjuvant:PBS (pH 7.2) emulsion followed by one or two booster injections (2 mg) on days 20 and 45 after the first immunization. Antibody titers were determined from test bleed using an ELISA procedure.

2.4. *Competition test in ELISA*

ELISA was carried out as previously described [17] with some modifications. Briefly, 50 μl of PheG1 B or PheG1 B-3 dissolved in absolute ethanol/hexane (1:1) at a concentration of 25 and 5 μl/ml, respectively, were coated by evaporation at 37°C overnight onto wells of microtitre plates (Nunc-Immuno Plate I). The ELISA competition test was done by incubating antisera at the indicated dilutions in wells of microtitre plates with potential inhibitor. The inhibitory glycolipids were thoroughly suspended at various concentrations in PBS + sodium deoxycholate (1 mg/ml) + 0.4% powdered defatted milk. Inhibitory sugar solutions were made in PBS + 0.4% powdered defatted milk. 90 μl of a serial dilution of test antigen or test methyl glycoside and 10 μl of a 10/X dilution of antiserum were added to each well (X = 100 for the PheG1 B antiserum and 50 for PheG1 B-3 antiserum) for 2 h at 37°C. After washings, anti-rabbit Ig horseradish peroxidase conjugate (Amersham France, Les Ulis, France) diluted 1 in 1,000 in PBS + 1% BSA was added followed by a 2 h incubation at 37°C. After washing three times, 100 μl of a solution of 2,2' azinobis (3-ethylbenzthiazoline sulfonic acid) at a concentration of 1 mg/ml and 0.01% H_2O_2 in acetate buffer were added to each well. Absorbance was read at 405 nm with a Multiskan apparatus (Flow Laboratories Inc., McLean, VA, USA).

The remaining activity percentage was calculated as follows:

$$100 \times \frac{A_{405}\,B - A_{405}\,C}{A_{405}\,D - A_{405}\,E}$$

where B = absorbance with inhibitor and coated antigen, C = with inhibitor and without coated antigen, D = without inhibitors and with coated antigen and E = without inhibitor and without coated antigen.

2.5. *Molecular modelling*

The representations of the carbohydrate parts of the phenolic glycolipids (Figs. 2 and 4) were drawn using the Alchemy program (Tripos Associates). The site cavity sizes of the anti-PheG1 B and anti-PheG1 B-3 antibodies were modelized from the CPK models of their respective antigen.

3. RESULTS AND DISCUSSION

3.1. *Anti-PheG1 B antibodies molecular specificity*

In order to determine the molecular specificity of the anti-Phe-G1 B antibodies, their binding to other mycobacterial phenolic glycolipids was investigated. The phenolic glycolipid from *M. kansasii* (PheG1 K-I), the phenolic glycolipids B-1, B-2 and B-3 from *M. bovis* BCG and the phenolic glycolipid from *M. marinum* (PheG1 M) were selected (their complete structures are summarized in Table I).

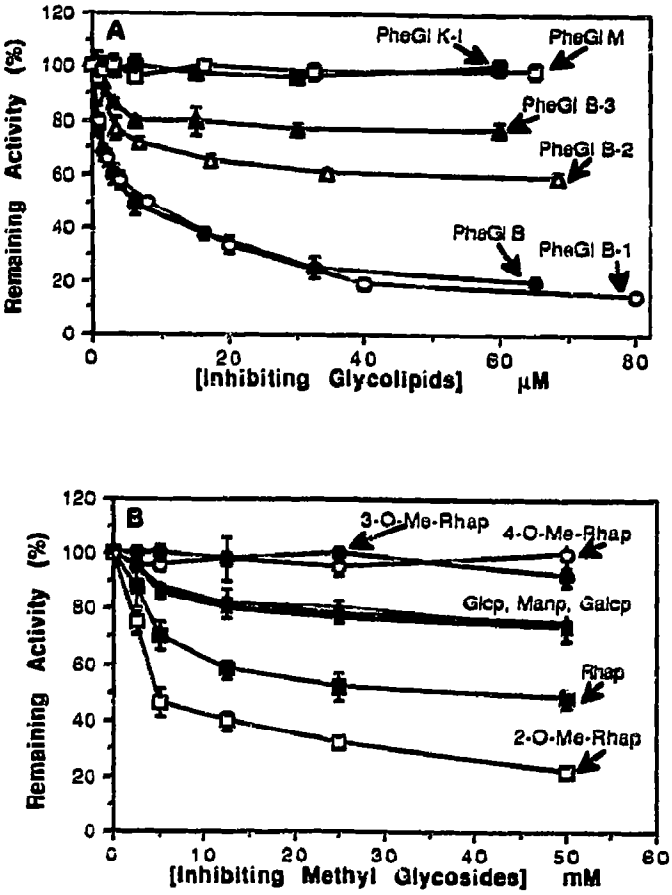


Fig. 1. Inhibition ELISA of PheG1 B antiserum binding to coated PheG1 B by (A) phenolic glycolipids B, B-1, B-2 and B-3 from *M. bovis* BCG, PheG1 M from *M. marinum* and PheG1 K-I from *M. kansasii* or by (B) methyl glycosides: methyl α-L-Rha*p*, methyl 2-*O*-Me-α-L-Rha*p*, methyl 3-*O*-Me-α-L-Rap*h*, methyl 4-*O*-Me-α-L-Rha*p*, methyl α-D-Glc*p*, methyl α-D-Man*p* and methyl α-D-Gal*p*. Following coating of PheG1 B, anti-PheG1 B serum diluted 1 in 100 (final dilution) was incubated with varying concentrations of inhibiting glycolipids or methyl glycosides for 90 min. The amount of antibodies bound was determined. Each point represents the arithmetic mean for three determinations.

Binding studies by competition ELISA (Fig. 1A) reveal that PheG1 B-1 binds to antibody with the same affinity as PheG1 B suggesting that the methoxyl group of the aglycone part is not involved in the binding process. Moreover, the complete loss of PheG1 K-I binding clearly demonstrates the absence of involvement of the aliphatic chain and consequently the delineation of the PheG1 B epitope to the 2-*O*-Me-α-L-Rha*p*. These results extend the previous data showing that *M. leprae* and *M. kansasii* phenolic glycolipidic epitopes are delineated to the carbohydrate part and more precisely to the disaccharidic unit located at the non-reducing end [19–21].

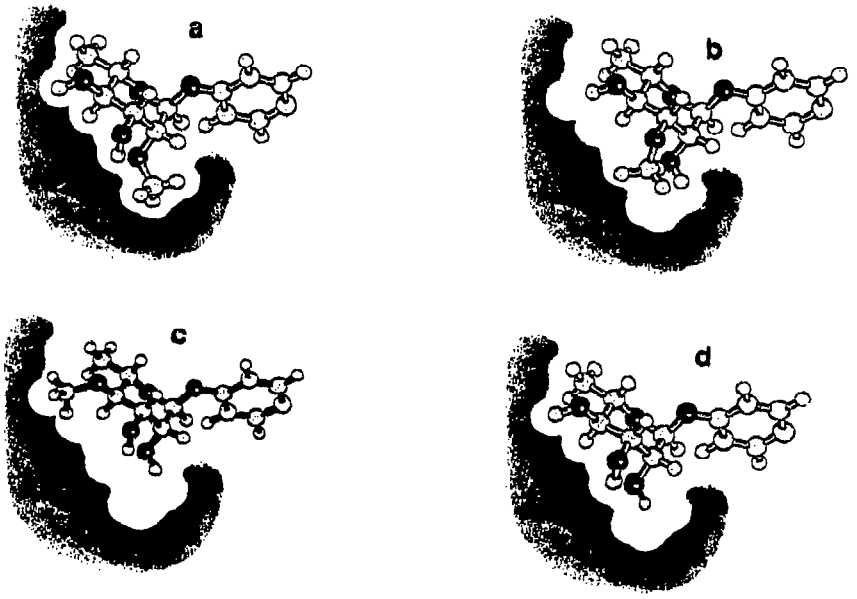


Fig. 2. Schematic representation of the binding site of the anti-PheG1 B antibody with the following ligands:
(a) 2-*O*-Me-α-L-Rha*p* 1→ *O* phenyl
(b) 3-*O*-Me-α-L-Rha*p* 1→ *O* phenyl
(c) 4-*O*-Me-α-L-Rha*p* 1→ *O* phenyl
(d) α-L-Rha*p* 1→ *O* phenyl

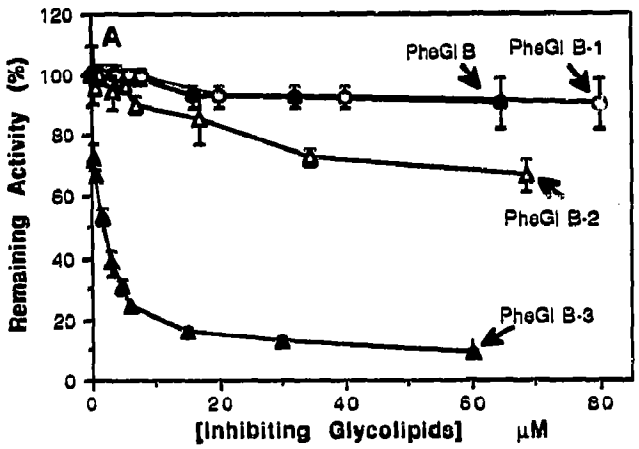
The PheG1 B-2 ligand significantly binds the anti-PheG1 B antibodies through its α-L-Rha*p*, while a complete loss of binding appears for the PheG1 M ligand. PheG1 B-3 shows a lower affinity than PheG1 B-2 although they share the same monosaccharide, α-L-Rha*p*, at the non-reducing end.

To determine the specificity of 2-*O*-Me-α-L-Rha*p* binding, methyl glycosides of Rha*p* with methoxyl groups at C-3 or C-4 were selected together with methyl α-L-Rha*p*. As expected, the binding studies show that methyl 2-*O*-Me-α-L-Rha*p* optimally binds the anti-PheG1 B antibodies in agreement with the fact that this glycoside residue is the PheG1 B epitope (Fig. 1B). Nevertheless, it can be observed that the methyl 2-*O*-α-L-Rha*p* affinity is 500-times lower than that of Phe G1 B. Similar results have been reported by Fujiwara et al. [21] concerning the binding of the phenolic glycolipid from *M. leprae* and their related monosaccharide and dissacharide epitopes to lepromatous leprosy sera. Unlike methyl glycoside epitopes glycolipid antigens have an amphipatic structure which allows micelle formation in aqueous solution. The difference in antibody affinity

Table 1

Structures of the phenolic glycolipids

Phenolic glycolipids from	Carbohydrate structure	Phenolglycol structure	Fatty acid structure
M. bovis BCG			
PheG1 B	2-*O*-Me-α-L-Rha*p*	Phenolphthiocerol	Mycocerosic C_{26}, C_{27}, C_{29}, C_{30}.
PheG1 B-1	2-*O*-Me-α-L-Rha*p*	Phenolphthiodolone	Mycocerosic C_{16}, C_{18}, C_{24}, C_{26}.
PheG1 B-2	α-L-Rha*p*	Phenolphthiodolone (*major homolog*)	Mycocerosic C_{26}, C_{27}, C_{29}.
PheG1 B-3	α-L-Rha*p* (1→3) 2-*O*-Me-α-L-Rha*p*	Phenolphthiocerol	Mycocerosic C_{26}, C_{27}, C_{29}.
M. kansasii			
PheG1 K-1	2,6-dideoxy-4-*O*-Me-α-D-arabino-hex*p*-(1→3)-4-*O*-Ac-2-*O*-Me-α-L-Fuc*p*-(1→3)-2-*O*-Me-α-L-Rha*p*-(1→3)-2,4-di-*O*-Me-α-L-Rha*p*	Phenolphthiocerol	Mycocerosic C_{29}, C_{30}, C_{32}.
M. marinum			
PheG1 M	3-*O*-Me-α-L-Rha*p*	Phenolphthiocerol	Mycocerosic C_{24}, C_{27}, C_{30}.



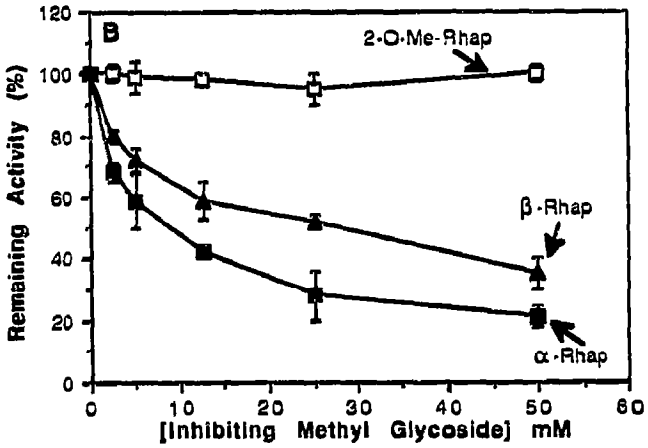


Fig. 3. Inhibition ELISA of PheG1 B-3 antiserum binding to coated PheG1 B-3 by (A) phenolic glycolipids B, B-1, B-2 and B-3 from *M. bovis* BCG or by (B) methyl glycosides: methyl α-L-Rha*p*, methyl β-L-Rha*p* and methyl 2-*O*-Me-α-L-Rha*p*. Conditions are the same as in Fig. 1 except that anti-PheG1 B-3 serum was diluted 1 in 50 (final dilution).

for these two types of molecules is likely to arise from better accessibility of carbohydrate parts of glycolipids at the micelle surface. Thus in order to discuss these respective binding specificities, one can only analyse the relative affinity values of the methyl glycoside ligands on one side or those of glycolipids on the other. Methyl α-L-Rha*p* also binds the anti-PheG1 B antibody but with a lower affinity than methyl 2-*O*-Me-α-L-Rha*p*. The complete loss of binding for the methyl 3-*O*-Me-α-L-Rha*p* are more striking and are in agreement with the unbinding of the PheG1 M, and the methyl 4-*O*-Me-α-L-Rha*p*. From these results it emerges that ligand unbinding is restricted to the location of the methoxyl group on the α-L-Rha*p* unit.

Considering the apolar nature of the phenolic glycolipids it can be assumed that hydrophobic interactions play a key role in promoting the stereospecific binding. It can be suggested that this process occurs through the presence of a cavity in the anti-PheG1 B immunoglobulin whose size can be estimated from the Van der Waals radius of the PheG1 B epitope: 2-*O*-Me-α-L-Rha*p*. Thus, the binding of the unmethoxylated Rha*p* can be explained, as illustrated in Fig. 2, by the decrease of the steric hindrance due to the substitution of a C-2 methoxyl group (PheG1 B) by a hydroxyl group (PheG1 B-2). Moreover, the lower affinity of PheG1 B-3 compared to PheG1 B-2 indicates that the penultimate monosaccharide of PheG1 B-3 is the cause of this effect by increasing the steric hindrance of the terminal monosaccharide unit. As shown in Fig. 2 steric hindrance appears to be the major factor of the unbinding of methyl 3-*O*-Me-α-L-Rha*p*. From these data, it can be speculated that a methyl glycoside ligand having a volume smaller than that of methyl 2-*O*-Me-α-L-Rha*p* must be able to bind the anti-PheG1 B antibodies with a lower affinity. This is the case of methyl α-L-Rha*p*, but also of methyl α-D-Man*p*, α-D-Glc*p* and α-D-Gal*p* (see Fig. 1B).

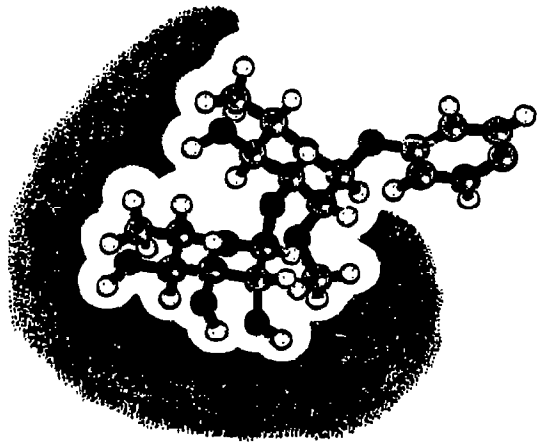


Fig. 4. Schematic representation of the binding site of the anti-PheG1 B-3 antibody with α-L-Rha*p* (1→3)-2-*O*-Me-α-L-Rha*p* 1→*O* phenyl.

It has been shown that hydrogen bonds also play a key role in carbohydrate antigen–antibody binding. Both the C-3 and C-4 hydroxyl groups of 2-*O*-Me-α-L-Rha*p* can participate in hydrogen bonding by either H donation or H acceptance from the protein. Thus the spatial orientation of both C-3 and C-4 hydroxyl groups, resulting from the arrangement of the 2-*O*-Me-α-L-Rha*p* epitope in the immunoglobulin cavity site, plays a key role in the stereospecific binding. The position of the 2-*O*-Me-α-L-Rha*p* is mainly controlled by the Van der Waals interactions which are proportional to r^{-6} (r, distance). The lower volume of α-L-Rha*p*, calculated from the Van der Waals radius, compared to the cavity size of the anti-PheG1 B antibody, led to a higher freedom, suggesting that both C-3 and C-4 hydroxyl groups are not optimally pointed to establish hydrogen bonds with the immunoglobulin amino acids. Such a process explains the lower affinity of α-L-Rha*p* binding compared to that of 2-*O*-Me-α-L-Rha*p*.

From these data two major parameters emerge for understanding the specific binding process of apolar glycolipids by antibodies: the Van der Waals volume of the immersed epitope ligand compared to the cavity size of the binding site, and the stereospecific linkage by hydrogen bonding. Thus it can be speculated that carbohydrate epitopes of lipopolysaccharides [22] and phenolic glycolipids [6] containing dideoxyhexoses at their non-reducing end will generate antibodies of higher molecular specificity.

3.2. *Anti-PheG1 B-3 antibody molecular specificity*

In a similar fashion, the binding process between PheG1 B-3 and anti-PheG1 B-3 antibodies was investigated (Fig. 3A). The optimally binding ligand is the PheG1 B-3 (estimated affinity 1 μM), and as expected the PheG1 B-3 epitope is delineated in the sugar part, α-L-Rha*p*-(1→3)-2-*O*-Me-α-L-Rha*p*. The anti-PheG1 B-3 antibodies also bind with a lower affinity to PheG1 B-2, whose carbohydrate structure is the α-L-Rha*p* unit but is unable to bind to PheG1 B.

Binding studies of the synthetic methyl glycosides (Fig. 3B) show that the α- and β-anomers of methyl L-Rha*p* optimally bind the anti-PheG1 B-3 antibodies while methyl 2-*O*-Me-α-L-Rha*p* binding must be so weak that our method can barely quantitate it. From these data it emerges that the α-L-Rha*p* unit located at the non-reducing end of PheG1 B-3 binds an immunoglobulin subsite with a higher affinity. However the substantial decrease of PheG1 B-2 binding suggests that the penultimate monosaccharidic unit, 2-*O*-Me-α-L-Rha*p*, is also involved in PheG1 B-3 binding. From these data, and by analogy with the monoclonal anti-dextran binding site [16] it can be proposed that the anti-PheG1 B-3 antibodies possesses two subsites, one with the major affinity and binding to the terminal monosaccharide unit (subsite I) while the other (subsite II), binds to 2-*O*-Me-α-L-Rha*p* with a lower affinity which can not be quantitated by the technique used (Fig. 4).

It can also be noticed that the affinity of the glycolipid B-3 for the anti-PheG1 B-3 antibodies is approximately six-times higher than that of PheG1 B for its antibodies. This can be explained by the fact that antibody binds optimally to two sequential glycosyl residues.

Finally it is interesting to consider that mostly oligoclonal antibodies are generated by the rabbit immune system when purified phenolic glycolipids such as PheG1 B and PheG1 B-3 were injected. Effectively, one could easily define a single epitope, delineated at 2-*O*-Me-α-L-Rha*p* unique sugar on the PheG1 B antigen, by the occurence in the whole serum of immunoglobulins that only bind the sugar part. Therefore it is obvious to conclude that these glycolipid antigens have structural regions rendered cryptic (the aglycon chain), together with obligatory antigenic determinant (the oligosaccharide), to the immune system.

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