Production and characterization of peptide mimotopes of phenolic glycolipid-I of *Mycobacterium leprae*

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Abstract

Phenolic glycolipid-I (PGL-I), a *Mycobacterium leprae*-specific antigen, has been widely used for the serodiagnosis of leprosy and has been implicated in the pathogenesis of leprosy. In an effort to produce an alternate antigen of PGL-I, the mimotope peptides of PGL-I, W(T/R)LGPY(V/M), were obtained using a monoclonal antibody, III603.8, specific to PGL-I by a phage library. The biotin-labeled predominant mimotope peptide of PGLP1, WTLGPYV, bound to III603.8 in a dose-dependent manner in an immunoassay. However, PGLP1 did not bind to anti-PGL-I antibodies in the serum samples from leprosy patients that were reactive to PGL-I. Although the mimotope peptide of WTLGPYV was not effective as an alternate antigen of PGL-I for the serodiagnosis of leprosy, but it would be of interest to know how the mimotope peptides mimic the role of PGL-I antigen in the pathogenesis of *M. leprae* infection.

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1. Introduction

Leprosy is still a major public health problem in several countries [1], and more than 500,000 new cases are reported worldwide each year. The early and rapid diagnosis of leprosy is a high research priority, because it would allow the effective control of leprosy in endemic areas. Accordingly, serological tests have been developed for the early detection of *Mycobacterium leprae* infection. Among the *M. leprae* antigens, phenolic glycolipid (PGL)-I has been most widely used for the serodiagnosis of leprosy because of its specificity [2].

Moreover, anti-PGL-I antibody levels are correlated with bacterial loads in leprosy patients. The sensitivity of PGL-I antigen-based serological tests ranges from 30% to 50% among paucibacillary (PB) patients to over 90% among multibacillary (MB) patients [3–5]. The prevalence of antibodies to PGL-I antigen among healthy controls depends on the leprosy burden in the geographical area, which demonstrates the usefulness of the PGL-I antigen-based serological test for measuring *M. leprae* transmission in a population of interest [6]. In addition, the PGL-I antigen has been used in serological testing for monitoring the treatment of leprosy patients, because of its rapid decline in serum following multidrug treatment [7,8].

PGL-I, the *M. leprae*-specific antigen, contains a unique trisaccharide, 3,6-di-O-methyl-β-D-glucopyranosyl-
Fig. 1. Structure of PGL-I of *M. leprae*. The trisaccharide 3,6-di-O-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-O-methyl-α-L-rhamnopyranosyl-(1→2)-3-O-methyl-α-L-rhamnopyranose is linked to phenol ring, which is linked to one of three tetramethyl-branched mycocerosic acids [9]. Recognition sites of the III603.8 and VI703 were terminal disaccharide, and the inner monosaccharide has some contribution to the reactivity of both mAbs.

(1→4)-2,3-di-O-methyl-α-L-rhamnopyranosyl-(1→2)-3-O-methyl-α-L-rhamnopyranose (Fig. 1) [2,9]. Native PGL-I antigen is very limited in terms of its availability because *M. leprae* can grow in vivo only in armadillo and mouse footpads [10,11]. In addition, it is difficult to use the PGL-I glycolipid antigen in immunoassays including enzyme-linked immunosorbent assay (ELISA) due to its strong hydrophobicity. Since early studies showed that the carbohydrates of the PGL-I antigen are critical for its binding to antibodies, a series of neo-glycoconjugate antigens containing carbohydrate epitopes of PGL-I were prepared and found to give a strong correlation with the native antigen in terms of detecting antibodies [12–14]. Currently, neoglycoconjugate antigens such as ND-O-BSA and ND-P-BSA are widely used for detecting anti-PGL-I antibodies [13,15]. Organic synthesis of the terminal disaccharides, 3,6-di-O-Me-Glc-(1→4)-2,3-di-O-Me-Rha, and the linking of this sugar to BSA carrier protein is, however, a tedious process requiring more than 20 steps [13].

One of the alternate strategies for replacing native PGL-I antigen involves the preparation of mimotope peptides resembling the B-cell epitope of the PGL-I antigen. Peptide mimicry using phage display technology to find a mimotope peptide has rapidly become a powerful tool for elucidating the conformation of the antigen (Ag) binding site and for providing molecular mimotopes of particular epitopes [16,17] for vaccine development. Numerous reports have suggested that molecular mimotopes of carbohydrate epitopes have immunogenic as well as antigenic properties [18,19]. Recently, peptide mimetic ligands have been used for its paratope-specific purification [20,21], but no report is available on the possibility of application of mimotope peptide to serodiagnosis. In this study, therefore, we prepared mimotope peptide resembling a carbohydrate epitope of the PGL-I antigen as an alternate source of antigen by using a monoclonal antibody (mAb), III603.8. However, its mimotope peptide was not efficient in detecting of anti-PGL-I antibodies in the sera of leprosy patients.

2. Materials and methods

2.1. Antigens

*Mycobacterium leprae*, PGL-I from *M. leprae*, deacylated PGL-I (PGL-I OH⁺), phenolic glycolipid-Tb (PGL-Tb) from *Mycobacterium tuberculosis*, mycoside A of *Mycobacterium kansasii*, deacylated mycoside B of *Mycobacterium bovis* and phthiocerol dimycocerosic acid (DIM) were kindly provided by Dr. P.J. Brennan (Colorado State University, Fort Collins, CO).

2.2. Production of mAbs

BALB/c mice were i.p. immunized with 50 μg of PGL-I and 50 μg of heat-killed *M. leprae* in the presence of incomplete Freund’s adjuvant, three or four times at 2- to 4-week intervals. Splenocytes were harvested and fused to X63-Ag8.653 myeloma cells using standard protocol described elsewhere [22]. Hybridoma supernatants were screened for reactivity to PGL-I by ELISA, and the cells that produced specific antibodies to PGL-I were expanded and subcloned by limiting dilution. The isotypes were determined by ELISA using isotype-specific polyclonal Abs (Roche Diagnostics GmbH, Mannheim, Germany).

For hybridoma screening, U-bottom microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with 50 μl of PGL-I dissolved in absolute ethanol (5 μg/ml) and evaporated at room temperature. After washing four times with phosphate-buffered saline solution (PBS, pH 7.4), the unbound surfaces of the wells were blocked with 5% normal goat serum (NGS)-PBS. Culture supernatants were added to the coated microtiter wells and incubated for 1.5 h at 37 °C. After washing, peroxidase-labeled anti-mouse immunoglobulins (Sigma Chemical Co., St. Louis, MO) and a substrate solution of o-phenylenediamine were employed in the assay. The reaction was stopped with 2.5 N sulfuric acid, and the optical densities were measured at 490 nm using an ELISA reader (Molecular Devices, Palo Alto,
CA). Two mAbs, III603.8 and VI703, were obtained in this study.

2.3. Purification of mAb and production of phage clones expressing the mimotopes

III603.8 mAb (IgM) from mice ascites was purified by using Sephaeryl S-300HR (Pharmacia, Uppsala, Sweden) after (NH₄)₂SO₄ precipitation and was used for biopanning the phage library. Phage libraries containing a linear peptide with seven amino acids from the New England Biolabs Inc. (Beverly, Maine) were used in this study. The biopanning of phage libraries and binding assays of phages has been described previously [22], and were used with minor modifications. Briefly, mAb was added to 60 mm dishes (Nunc, Denmark) at a concentration of 100 μg ml⁻¹ in 0.1 M NaHCO₃ (pH 8.6) and incubated overnight at 4 °C. The dishes were then washed with 0.1% Tween 20–TBS (TBST; 50 mM Tris–Cl, pH 7.5, 150 mM NaCl) after blocking with 0.5% BSA in 0.1 M NaHCO₃. Phages, 2 × 10¹¹ plaque-forming units (pfu), were biopanned three times, and bound phages were eluted with 0.2 M glycine–HCl (pH 2.2) and neutralized with 1 M Tris–HCl (pH 9.1). After amplification, individual phage clones were examined for binding characteristics, and their nucleotide sequences were determined to deduce the mimotope peptides.

2.4. Binding assays of phages to mAb

III603.8 mAb was absorbed in the wells of 96-well ELISA plates (Nunc) at a concentration of 10 μg ml⁻¹ in carbonate–bicarbonate buffer (pH 9.6). After blocking mAb-coated wells with 5% NGS-PBST, amplified phages were added to the wells at 4-fold dilutions of the original stock phages (or 6 × 10¹⁰ pfu/well) and incubated at room temperature for 1.5 h. Subsequently, peroxidase-labeled anti-phage mAb (Pharmacia) was used for detecting bound phages, and O-phenylenediamine was used as a substrate for peroxidase enzyme.

Competitive inhibition ELISA was employed to determine any specific binding of phages to mAb III603.8. Briefly, native PGL-I antigen was coated on the wells of ELISA plates as described above. III603.8 was first mixed with phages in serial 10-fold dilutions with 5% NGS-PBS from the original stock of phages and then added to the PGL-I-coated wells. The mixture was then incubated at room temperature for 2 h with gentle agitation. Peroxidase-labeled anti-mouse Ig was used as a secondary Ab. Finally, the optical density was measured at 490 nm as described above.

2.5. Sequencing DNA encoding phage peptide

Phage clones were expanded by growing Escherichia coli transfected with phages in 1 ml of LB broth at 37 °C for 4–5 h to determine the DNA sequences encoding the mimotope peptide. To recover the phages, the bacteria were first removed by briefly centrifuging the culture at 10,000 g for 30 s. About 500 μl of the culture supernatant was mixed with 200 μl of 20% PEG/2.5 M NaCl solution, and the mixture was centrifuged at 10,000 g for 10 min. The pellet containing the phages was resuspended in a solution consisting of 100 μl of a Tris buffer with iodide (10 mM Tris–Cl, pH 8.0, 1 mM EDTA, 4 M NaI) and 250 μl of absolute ethanol. The phages were then washed with 70% ethanol, dried, and resuspended in 30 μl of TE buffer. Five microliters of the phage suspension was subjected to dideoxy termination reaction using a DNA sequencing kit (Perkin–Elmer, Norwalk, Conn., –96 sequencing primer, and AmpliTaq DNA polymerase FS (Applied Biosystems, Foster City, CA). The sequences of DNA encoding the mimotope were analyzed by running the above reaction products through an automated DNA sequencer from Applied Biosystems (Foster City, CA).

2.6. Binding of anti-PGL-I antibodies to biotin-PGLP1

A phage clone containing the peptide sWTLGYPVg was most frequently found among the phage clones to III603.8 and was designated PhaPGLP1 (Table 1). Phage protein pH1 flanking the peptide insert are shown in small letters. For serologic testing, the biotin-labeled peptide KGGSWTLGYPVG (biotin-PGLP1) was synthesized (Peptron Co., Daejon, Korea). The peptide KGG was added to the insert peptide and designated as PGLP1. To determine the binding activity of the PGLP1 mimotope peptide to serum samples, microtiter plates were precoated with streptavidin at a concentration of 10 μg ml⁻¹ for overnight and washed with PBST. Biotin-PGLP1 was added to the wells at given concentrations for 2 h at 37 °C, and binding was performed with 5% NGS-PBST after washing. Two known leprosy serum samples were added to the wells at fixed dilution and incubated at room temperature for 3 h. III603.8 was used as a positive control antibody. After washing the wells, peroxidase-labeled anti-mouse Ig was added, and the peroxidase activity was quantitated with O-phenylenediamine substrate as described above.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Mimetic peptide sequences of III603.8</th>
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<tr>
<td>Phage</td>
<td>Peptide sequences³</td>
</tr>
<tr>
<td>PhaPGLP1</td>
<td>sWTLGYPVg</td>
</tr>
<tr>
<td>PhaPGLP2</td>
<td>sWRLGYPVg</td>
</tr>
<tr>
<td>PhaPGLP3</td>
<td>sWTLGYPVm</td>
</tr>
</tbody>
</table>

³Sequences of clones from heptapeptide insert phage library after third biopanning by III603.8 are shown. Flanking sequences are shown in small letters and inserted sequences are shown in capital letters.

²Number of identical peptide sequence.
3. Results and discussion

3.1. Antigenic specificities of mAbs

In this study, we produced and characterized mAbs to PGL-I and found the mimotope peptide resembling the carbohydrate epitope of the PGL-I antigen (Fig. 1) as an alternate source of the antigen by using III603.8 mAb, and tested whether the mimotope peptide could be useful to apply the serodiagnosis of leprosy.

Two clones of hybridoma, III603.8 and VI703, were established and their isotypes were identified as IgM and IgG3 with kappa light chains, respectively. These two clones bound to PGL-I and deacylated PGL-I (PGL-I OH⁻/C₀⁻), but did not bind to PGL antigens from M. kansasii (mycoside A), M. bovis (deacylated mycoside B), and M. tuberculosis (PGL-Tb OH⁻) (Fig. 2(a)). The terminal sugar of 3,6-di-O-methyl-β-D-glucopyranose could not inhibit the binding of the selected phage clone of PhaPGLP1 (Table 1) to III603.8 (data not shown). Both mAbs bound to the plates coated with NT-O-BSA, a trisaccharide-containing neoglycoconjugate, better than with ND-O-BSA, a terminal disaccharide-containing neoglycoconjugate, at the same concentration of antigen coating (Fig. 2(b)).

The analysis of recognition sites for the trisaccharides of PGL-I using 12 mAbs suggested that there are at least five epitopes in the trisaccharides [23]. Although there was some ambiguity concerning each epitope, the epitopes of the PGL-I antigen included the outer monosaccharide, the outer disaccharide, the trisaccharide, the inner disaccharide, and the inner monosaccharide-containing structure [23]. These data suggest that both of our mAbs bind specifically to PGL-I and that all three carbohydrates of PGL-I antigen contribute to the binding of PGL-I to both mAbs. We used III603.8 for further analysis of the mimotope peptide to PGL-I.

3.2. Selection of phage clones

For the selection of phage clones containing mimotope peptide insert to PGL-I, we purified III603.8 mAb, which was produced from mouse ascites. After the third round of biopanning a phage library displaying random linear peptides, a total of 24 separate phage clones were randomly chosen and amplified. Twenty-two clones bound to III603.8 and all these phage clones inhibited the binding of III603.8 to PGL-I. The reactivities of two representative phage clones containing different peptide inserts, PhaPGLP1 and PhaPGLP2, are shown in Fig. 3. Fig. 3(a) shows that PhaPGLP1 and PhaPGLP2 bound to III603.8 in a dose-dependent manner, and Fig. 3(b) shows that the two phage clones inhibited the binding of III603.8 to PGL-I antigen, but that irrelevant phage...
PhaControl) showed no inhibition even at its highest level. So it was considered that both phage clones contained the peptide inserts that were mimic to the region of PGL-I antigen that III603.8 was bound.

3.3. Analysis of the peptide motif sequence

The nucleotide sequences of the inserted DNA of 22 cloned phages were determined, and the amino acid sequences were translated. Among the 22 phage clones obtained with III603.8, 18 (81.8%) clones were found to express the sequence WTLGPYV, represented by the PhaPGLP1 clone (Table 1). Two additional sequences of WRLGPYV (3 clones, 13.6%) and WTLGPYM (1 clone, 4.6%), represented by PhaPGLP2 and PhaPGLP3, respectively, were also identified (Table 1). Among the consensus sequences of W(T/R)LGPY(V/M) found in all 22 phage clones, and T, a polar amino acid, was replaced by R in three clones and V was replaced by M, another hydrophobic amino acid in one clone. The binding of mimotope peptide to antibody paratope is controlled by three-dimensional conformation and interactions between the peptide and complementarity-determining regions of antibody such as ionic bond and hydrophobic binding [20]. These consensus sequences were not previously reported although there is a possibility of finding further mimotope peptide sequences to III603.8 mAb. All mimotope peptide sequences identified in this study contained two aromatic acids, and this finding have been found in the mimotopes of many polysaccharides such as meningococcus group B [22,24] and group C [25], group B streptococcal type III [26], and pneumococcal capsular PS of 6B serotype [24].

3.4. The mimotope peptide was bound to III603.8 but not to anti-PGL-I antibodies of leprosy serum samples

To investigate whether the peptide inserts were responsible for the binding of phage particles to III603.8, we synthesized the mimotope peptide of PGLP1, WTLGPYV, and conjugated the peptide with BSA by glutaraldehyde method. However, the seroreactivities to the PGLP1-BSA conjugate showed unexpectedly high nonspecific binding values when we measured the binding to PGLP1-BSA using serum samples from leprosy patients and normal individuals and using even III603.8 mAb (data not shown). When we compared the binding of two known serum samples to PGL-I, ND-O-BSA or biotin-PGLP1, the serum samples were well bound to PGL-I and ND-O-BSA at 1:300 dilution but very poor binding to biotin-PGLP1 even at 1:50 dilution (Fig. 4(b)). We could think a couple of reasons that anti-PGL-I antibodies in leprosy serum samples showed the poor binding to biotin-PGLP1. First, anti-PGL-I antibodies in sera from leprosy patients were composed of polyclonal antibodies to heterogeneous epitopes of PGL-I. Since the outer monosaccharide of 3,6-di-O-methyl-glucose
was required for the reactivity of PGL-I to the sera of leprosy patients [29], there were at least three epitopes that contain 3,6-di-O-methyl-glucose in the PGL-I trisaccharide. Thus it is reasonable to believe that serum samples from leprosy patients contain antibodies to more than one epitope of the PGL-I antigen. In order to detect most of the anti-PGL-I antibodies, therefore, mimotope peptides need to be prepared from monoclonal antibodies that cover as many epitopes of the PGL-I antigen as possible or from anti-PGL-I antibodies purified from serum samples of leprosy patients. Mixtures of such mimotope peptides would give a better reaction to the sera of leprosy patients than the PGL1 peptide of this study. Second, III603.8 is not to the major target epitope of PGL-I that can induce anti-PGL-I antibodies in leprosy patients. And anti-PGL-I antibodies in sera from leprosy patients may bind to synthetic peptide of biotin-PGL1 with very low affinity. The phage-derived mimotope peptides have relatively low affinity, and affinity maturation of mimotope peptide by substitution of an amino acid or by use of D-amino acid increases the binding of mimotope peptide to mAb [20,30]. And considering that peptide length is an important factor in the design of immunoaffinity ligand [20] because of the increase of peptide mobility and forcing conformations, the additions of KGGS and G at both terminals to the mimotope peptide probably hindered antibody binding.

In summary, the phage display technology employed to identify mimotope peptides in this study should be useful in the preparation of a set of epitopes that mimic the carbohydrate immunodeterminants of PGL-I. The mimotope peptide of WTLGPYV prepared from III603.8 was found to be specific to the PGL-I antigen, but this mimotope peptide was not effective in detecting anti-PGL-I antibodies in the sera of leprosy patients. It would be of interest to know how the mimotope peptides mimic the role of PGL-I antigen in the pathogenesis of M. leprae infection, particularly in inducing cell-mediated immune responses.

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