Peptide mimotopes of *Mycobacterium tuberculosis* carbohydrate immunodeterminants

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Cell-surface saccharides of *Mycobacterium tuberculosis* appear to be crucial factors in tuberculosis pathogenicity and could be useful antigens in tuberculosis immunodiagnosis. In the present study, we report the successful antigenic and immunogenic mimicry of mannose-containing cell-wall compounds of *M. tuberculosis* by dodecamer peptides identified by phage-display technology. Using a rabbit antiserum raised against *M. tuberculosis* cell-surface saccharides as a target for biopanning, peptides with three different consensus sequences were identified. Phage-displayed and chemically synthesized peptides bound to the anti-carbohydrate antiserum. Rabbit antibodies elicited against the peptide QEPLMGTVPIRAGGGS recognize the mannosylated *M. tuberculosis* cell-wall antigens arabinomannan and lipoarabinomannan, and the glycosylated recombinant protein alanine/proline-rich antigen. Furthermore, antibodies were also able to

react with mannan from *Saccharomyces cerevisiae*, but not with phosphatidylinositol dimannosides or arabinogalactan from mycobacteria. These results suggest that the immunogenic peptide mimics oligomannosidic epitopes. Interestingly, this report provides evidence that, in contrast with previously known carbohydrate mimotopes, no aromatic residues are necessary in a peptide sequence for mimicking unusual glycoconjugates synthesized by mycobacteria. The possible usefulness of the identified peptide mimotopes as surrogate reagents for immunodiagnosis and for the study of functional roles of the native non-peptide epitopes is discussed.

Key words: glycoconjugate, immunodeterminant, lipoarabinomannan (LAM), mimotope, *Mycobacterium*, phage display.

INTRODUCTION

With extraordinarily high rates of morbidity and mortality worldwide and the dubious distinction as the only disease declared by the World Health Organization as a global emergency, tuberculosis remains one of the major health problems in the world. One third of the world's population is infected with *Mycobacterium tuberculosis*, which causes 2 to 3 million deaths per year. The molecular bases of tuberculosis disease are still unclear. We lack a full understanding about the biology of the tubercle bacillus, which has the ability to persist for long periods inside the host. However, it has been demonstrated that *M. tuberculosis* cell envelope is a key factor in tuberculosis pathogenicity. In particular, mycobacterial cell-wall sugars are involved in the early stages of mycobacterial infections [1] as well as in various subsequent interactions with host cells and tissues [2,3]. Ultrastructural techniques have recently provided evidence that the envelope surrounding mycobacteria is composed of multiple layers as visualized by electron microscopy; these layers correspond to a typical bacterial membrane, a wall and an outermost layer composed of a capsule-like material endowed with distinct saccharide compounds [1,2]. Frequently, these saccharides and glycoconjugates are complex and show structural heterogeneity within the same mycobacterial strain [2,3]. Thus, the identification of epitopes involved in the different biological functions of cell-envelope mycobacterial sugars is difficult, and the nature of these molecules as secondary genetic products makes their recombinant production unfeasible.

In the past decade, the phage-displayed random peptide libraries have emerged as a powerful technique to look for peptide mimics of sugars [4,5]. In the present study, this method was employed to identify mimotopes of cell-surface mycobacterial carbohydrates by screening a linear dodecapeptide library with rabbit antibodies raised against an *M. tuberculosis* cell-surface fraction enriched with NPS (neutral polysaccharides). A bioselection to identify phages carrying peptide inserts specifically binding to the serum was performed, and the expressed peptides were analysed for their ability to mimic mycobacterial cell-surface carbohydrates. Our results show that the screening resulted in the isolation of peptides mimicking mannose-containing compounds of *M. tuberculosis*, at both antigenic and immunogenic levels, and that mimicked epitopes comprise oligomannoside motifs also occurring in yeast glycoconjugates. In addition, we obtained evidence for the first time that the presence of aromatic amino acid residues is not necessary in a peptide sequence to mimic sugar compounds.

EXPERIMENTAL

Reagents

The desalted peptides P1 (QEPLMGTVPIRAGGGS), P2 (TLQQ-NMPLALVWGGGS) and P3 (DPEQPAPLFLVSGGGS) were purchased from Bio-Synthesis (Lewisville, TX, U.S.A.), and purified by reversed-phase HPLC on a Delta Pack C18, 5 μ m, 300 Å (1 Å = 10⁻¹⁰ m) column (300 mm × 39 mm; Waters, Mildford, MA, U.S.A.), equilibrated at a flow rate of 1.5 ml/min and eluted

Abbreviations used: ABTS, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); AM, arabinomannan; Apa, alanine/proline-rich antigen; ConA, concanavalin A; LAM, lipoarabinomannan; mAb, monoclonal antibody; NPS, neutral polysaccharides; PIM, phosphatidylinositol mannoside.

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with a linear gradient from 0 to 70% of CH₃CN in 0.1% trifluoroacetic acid on a Waters HPLC system equipped with a UV detector model 486 (Waters). Purified peptide having the unrelated sequence PELGASLGREYHSAR and phage clone APP-12 (3.7) expressing an un-related peptide with the sequence MTKDDVFHPDVY were used as controls. CS35 anti-LAM (lipoarabinomannan), mAb (monoclonal antibody), PIMs (phosphatidylinositol mannosides), arabinogalactan and mannose-capped LAM from *M. tuberculosis* were provided by Dr J. Belisle of Colorado State University under the terms of the NIH-sponsored Tuberculosis Research Materials and Vaccine Testing Contract (NO1, AI-75320). Recombinant 45/47 kDa Apa (alanine/proline-rich antigen) protein was expressed in *Streptomyces lividans* and purified by ConA (concanavalin A)-Sephacrose column chromatography as described previously [6]. Quality control of LAM and Apa preparation included blotting analyses with Coomassie Blue and ConA staining as well as immunoblotting with CS35 and 6A3 mAbs directed against LAM and Apa respectively. PIMs were analysed by TLC, developed with chloroform/methanol/water (65:25:4, by vol.) and revealed with anthrone and Molybdenum Blue reagent (Sigma, St. Louis, MI, U.S.A.). Mannan from *Saccharomyces cerevisiae* was purchased from Sigma.

NPS of *M. tuberculosis*

M. tuberculosis H37Rv was grown on Sauton mineral salts medium as surface pellicles at 37°C for 5 weeks. The culture filtrate, passed through a 0.22 µm membrane, was concentrated under vacuum to one-tenth of the original volume and NPS were obtained as described by Lemassu and Daffé [7]. Briefly, the concentrated filtrate was partitioned in chloroform/methanol/water (3:4:3, by vol.), and the aqueous phase was precipitated overnight at 4°C with 6 vol. of cold ethanol. The pellet was recovered after centrifugation at 14 000 g for 1 h, dissolved in distilled water, precipitated again with ethanol and dialysed against distilled water. NPS were obtained by DEAE-Trisacryl (Sigma) column chromatography. Carbohydrate-containing fractions, detected by anthrone reaction, were pooled and adjusted to 1.5 mg/ml. Proteolytic digestion of the NPS fraction was performed using 20 µg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) in 10 mM EDTA, 10 mM Tris, pH 8.0 for 3 h at 50°C, followed by an inactivation period of 2 h at 65°C. Neutral saccharide analysis of the NPS was performed by GLC, after acid hydrolysis (2 M trifluoroacetic acid at 120°C) and transformation of the released monosaccharides into their trimethylsilyl derivatives [8].

Antibodies

All experiments with animals were performed in accordance with institutional guidelines. For generation of polyclonal antibodies against the NPS fraction, two New Zealand white rabbits (2–2.5 kg) received subcutaneous injections of NPS (0.75 mg in 0.5 ml of PBS emulsified with an equal volume of Freund's adjuvant). The animals received booster injections (on day 7 and 14 after initial priming immunization) with 0.375 mg of NPS in 0.5 ml of PBS with 0.5 ml of incomplete Freund's adjuvant. Antibody titres were examined by ELISA at 0, 2, 3 and 4 weeks after the initial immunization and the animals were killed by cardiac exsanguination at the end of the fourth week. The titre of the anti-NPS antiserum used for biopanning was 1:3200. For generation of polyclonal sera against peptides, 2–3 month old New Zealand rabbits were immunized subcutaneously in two sites with 500 µg of the peptide dissolved in 500 µl of saline solution prepared with pyrogen-free water and emulsified with an equal

volume of incomplete Freund's adjuvant. Then, three boosters with 250 µg of peptide were injected at intervals of 3 weeks.

Phage display selection

A linear dodecamer random peptide library displayed on a filamentous phage M13 as a fusion to minor coat protein III (cpIII) was purchased from New England Biolabs (Beverly, MA, U.S.A.). The complexity of the library was approx. 1.9×10^9 transformants. Polyclonal anti-NPS rabbit hyperimmune serum was used for affinity selection, essentially as described by Gevorkian et al. [9]. Briefly, microplate wells (Nunc MaxiSorp, Roskilde, Denmark) were coated with 0.5 µg of rabbit anti-IgG (Zymed, San Francisco, CA, U.S.A.) diluted in 100 µl of PBS for 1 h at 37°C. After washing with PBS, 200 µl of 3.0% (w/v) BSA in PBS were added and plates were incubated for 1 h at 37°C. Then 100 µl of anti-NPS serum, diluted 1:100, were added. After an incubation period for 1 h at 37°C, wells were washed with 0.1% (v/v) Tween 20 in PBS, and 10^{11} phage particles from the dodecapeptide library were allowed to react in wells with the anti-NPS polyclonal antiserum for 2 h at 4°C and 1 h at 37°C. After incubation, unbound phages were discarded, wells were washed with PBS and bound phages were eluted with glycine/HCl (pH 2.2). Eluates were transferred to microfuge tubes and neutralized with 35 µl of 2 M Tris base. Eluted phages were amplified by infection of *Escherichia coli* ER2537 cells in 2 × YT medium (16 g of Bacto-Tryptone, 10 g of yeast-extract and 5 g of NaCl per litre; Gibco BRL, Gaithersburg, MD, U.S.A.) and incubation for 4 h with shaking at 37°C. Next, the cells were centrifuged at 12 000 g, the phages purified by double precipitation with polyethylene glycol (USB, Cleveland, OH, U.S.A.) and 10^{12} plaque-forming units were used for the next round of biopanning. After three rounds, individual phage clones were isolated. The phage ssDNA (ss, single-stranded) was purified as described by Sambrook et al. [10] and used for DNA sequencing, which was performed with the T7 Sequenase version 2.0 Quick-Denature plasmid sequencing kit (Amersham Biosciences, Cleveland, OH, U.S.A.), -96gIII sequencing primer (New England Biolabs) and [α -³⁵S]dATP (Amersham Biosciences), according to the manufacturers' instructions. The DNA and amino acid sequences of peptides were analysed by computer search with ExPASy molecular biology server (<http://www.expasy.ch/tools>). Consensus sequences were determined by multiple sequence alignment with ClustalX.

ELISA

To evaluate the binding of antibodies to the selected phage-displayed peptides, Maxisorp multiwell plates (Nunc) were coated with 100 µl of a 5 µg/ml solution of anti-Fc rabbit IgG mAb (Zymed) in PBS for 1 h at 37°C. After washing with 0.1% Tween 20 in PBS, the wells were incubated with 200 µl of 3% BSA in PBS for 1 h at 37°C. Anti-NPS antiserum was added, diluted at 1:400 (v/v) in 1% BSA in PBS, and incubated for 1 h at 37°C. Then, phage particles were suspended in 1% BSA in PBS and added to the wells (at 10^{10} plaque-forming units/ml). After a 3 h incubation period at 4°C and 30 min at room temperature (23°C), the plates were washed. Subsequently, capture of the phages by antibodies was detected with anti-M13 mAb conjugated to horseradish peroxidase (Zymed) using ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] solution (Zymed) as a substrate. The reaction was read at 405 nm. A similar method was used to determine the binding of the phage clones to human sera, except that anti-(human IgG) mAb (Zymed) was used instead of anti-Fc rabbit IgG mAb.

For monitoring the binding of antibodies to microbial antigens (NPS fraction, LAM, mannan, recombinant Apa protein and arabinogalactan) and synthetic peptides, the plates were coated with the compounds (1 or 2 µg/well for purified LAM) for 16 h at 4°C, washed and saturated with 2% BSA in PBS for 1 h at 37°C. After four washings with 0.2% Tween 20 in PBS, antibodies (anti-NPS, anti-P1 or CS35 mAb) were added to the wells, incubated for 1 h at 37°C and washed four times. Anti-rabbit IgG or anti-mouse IgG conjugated to peroxidase (Zymed) was added to detect the binding of antibodies. Antibodies from human sera were determined by a similar procedure, using 1:100 (v/v) serum dilutions and anti-(human IgG) conjugated to peroxidase. After four washings, the peroxidase activity was monitored by the addition of ABTS substrate solution at 37°C. Absorbance was recorded at 405 nm. The reactivity of antibodies to *M. tuberculosis* PIMs was determined using a previously reported ELISA method for glycolipid antigens [11].

Gel electrophoresis and immunoblotting

LAM-containing *M. tuberculosis* antigen preparation was obtained by homogenization and centrifugation of heat-inactivated bacterial cells. *M. tuberculosis* cells were washed with PBS/0.05% Tween 20, and resuspended (1:1, w/v) in 10 mM Tris/HCl (pH 7.5). The suspension was sonicated for 15 min, vortex-mixed with glass beads (106 µm diameter) for 5 min and centrifuged at 15000 g for 30 min at 4°C. The supernatant, rich in protein and LAM, was used for Western blotting. The protein content was estimated using a commercial protein assay reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Samples were analysed by SDS/PAGE (12.5% polyacrylamide) together with molecular-mass standards and stained by a silver-staining method (Bio-Rad Laboratories) or transferred on to nitrocellulose membranes in a Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad Laboratories). In some experiments, samples were treated with proteinase K using the method described above. The blots were washed in PBS and blocked for 3 h with 3% BSA in PBS. Strips of width 5 mm were incubated overnight at 4°C with antibodies (hyperimmune rabbit sera or CS35 anti-LAM mAb). Membranes were incubated with biotinylated Protein A (1:500, v/v), followed by incubation with streptavidin-peroxidase (1:500, v/v), and 3,3'-diaminobenzidine (Sigma) was used as a substrate. Glycosylated products were detected by using ConA conjugated to peroxidase (Sigma), subsequently incubated with 3,3'-diaminobenzidine and H₂O₂.

Human sera

Sera from ten individuals with pulmonary tuberculosis were gifts from Dr C. Hermida from the Hospital de Infectología del Centro Médico Nacional 'La Raza' in Mexico City. All patients were diagnosed by acid-fast smear or by positive culture. Positive reactivity of these sera with the NPS fraction was confirmed by indirect ELISA, as described above. Sera from ten healthy blood donors were from the Banco de Sangre del Centro Médico Nacional 'Siglo XXI', in Mexico City.

RESULTS

Production and characterization of anti-carbohydrate antibodies

New Zealand rabbits were immunized with an NPS fraction of *M. tuberculosis*, whose main sugar components, as determined by GLC, were arabinose and mannose (results not shown). After three immunizations, the rabbit sera showed a titre of 1:3600, as

Table 1 Peptide sequences, frequency and ELISA binding reactivity to anti-NPS antibodies of the selected phage clones

The binding reactivity of phage clones with anti-NPS antibodies was evaluated by a capture ELISA as described in the Experimental section. Frequency indicates the number of times each sequence was independently isolated. The fold rise in the ELISA A₄₀₅ values when compared with those obtained with the preimmune serum is indicated. Consensus amino acid residues are highlighted in bold.

Clone	Group	Amino acid sequence	Frequency	Rise in ELISA value (fold)
NSP-1	I	WSA P VLMGT V PP	8/19	8.16
NSP-2	I	RKV P LLSGTL P Q	1/19	9.52
NSP-3	I	QE P LMGT V PIRA	1/19	8.48
NSP-4	II	QLNKL Q IPL S II	2/19	7.03
NSP-5	II	THNH Q VPL A HS	1/19	7.83
NSP-6	II	TMPWN Q SALT I	1/19	5.57
NSP-7	IIB	TLQ Q N M P L AL V W	1/19	8.20
NSP-8	IIB	DPE Q AP L FL V S	1/19	11.31
NSP-9	IIB	API Q A H PLGLIR	1/19	9.80
NSP-10	III	PSI G GGSSVD L V	1/19	6.04
NSP-11	III	IDIINPAQ N RLR	1/19	8.47

assessed by ELISA using the NPS fraction as antigen. No loss of the titre was observed when the NPS fraction was initially treated with proteinase K. These results showed that the hyper-immune antiserum mainly includes the antibodies reacting against non-protein components from the NPS fraction of *M. tuberculosis*.

Selection of phage clones and analysis of peptide sequences

The anti-NPS polyclonal antibodies were used to screen a commercially available phage-displayed dodecapeptide library. After three rounds of biopanning, 20 individual clones were randomly selected and analysed. DNA sequences of the phage inserts were determined and one clone carrying an insert with similarity to an *M. tuberculosis* H37Rv open reading frame was discarded for this study. Sequences of peptide inserts from 19 phage clones are shown in Table 1. According to the sequences of their peptide inserts, phage clones were arranged into four groups. Group I, representing more than half of the selected clones, with the consensus sequence **P**LXGT[L/V]**P**; group II, with four clones displaying the consensus sequence **Q**X[P/A]**L**X[I/L]**I**; group IIB, with three clones expressing the consensus sequence **Q**XXPL-XL[V/I]; and group III, composed of clones which do not have any consensus sequence. After amplification of phage clones, ELISAs were performed to verify the recognition of phages by the anti-NPS antibodies. All selected clones were strongly recognized by the anti-NPS rabbit antiserum, showing comparable levels of binding (Table 1), and demonstrating that peptide inserts are antigenic mimotopes of the mycobacterial NPS fraction. When phage clones were used as antigens in ELISA with human sera, a stronger reactivity was found with sera from *M. tuberculosis*-infected patients compared with sera from healthy donors, and some clones showed specific responses with sera from infected patients. In contrast, close reactivity values were obtained with sera from both groups when a non-related phage clone was used as antigen (Table 2).

Antigenic and immunogenic properties of synthetic peptide mimotopes

To study immunological properties of mimotopes in the form of phage-free molecules, peptides based on amino acid sequences of three phage clones were chemically synthesized. Clones NSP-3, NSP-7 and NSP-8, which presented high levels of

Table 2 Reactivity of human sera with selected phage clones by a capture ELISA test

Human antibodies were immobilized to anti-(human IgG) mAb-coated microtitre plate wells. Phage clones were added and, after washings, the binding was developed with anti-M13 mAb conjugated to peroxidase and ABTS substrate solution. A_{405} values are means $\pm$ S.D. ($n=10$). False-positive responders are healthy individuals having antibody levels greater than the mean $+2$ S.D. A_{405} value obtained with control serum. A phage clone expressing a non-related peptide (NRP) was used as control.

Clone	Consensus group	A_{405}		False-positive responders
		<i>M. tuberculosis</i> -infected patients	Healthy controls	
NSP-1	I	0.37 $\pm$ 0.17	0.15 $\pm$ 0.05	0
NSP-2	I	0.61 $\pm$ 0.17	0.20 $\pm$ 0.06	1
NSP-3	I	0.52 $\pm$ 0.20	0.17 $\pm$ 0.07	0
NSP-4	II	0.46 $\pm$ 0.16	0.20 $\pm$ 0.12	1
NSP-5	II	0.60 $\pm$ 0.18	0.38 $\pm$ 0.11	1
NSP-6	II	0.67 $\pm$ 0.23	0.28 $\pm$ 0.11	1
NSP-7	IIb	0.36 $\pm$ 0.15	0.13 $\pm$ 0.05	0
NSP-8	IIb	0.43 $\pm$ 0.15	0.20 $\pm$ 0.06	0
NSP-9	IIb	0.42 $\pm$ 0.16	0.17 $\pm$ 0.04	0
NSP-10	III	0.69 $\pm$ 0.19	0.41 $\pm$ 0.12	0
NSP-11	III	0.18 $\pm$ 0.10	0.07 $\pm$ 0.09	2
NRP	—	0.18 $\pm$ 0.07	0.11 $\pm$ 0.04	—

Figure 1 Peptide motifs identified by biopanning experiments are antigenic mimics of the NPS fraction from *M. tuberculosis*

Microtitre plate wells were coated with synthetic peptides or NPS (1 μ g of peptide or sugars/well). After blocking and washing the wells, preimmune rabbit serum (open bars) or anti-NPS hyperimmune rabbit antiserum (black solid bars) was added. After incubation, the reaction was developed with anti-rabbit IgG conjugated to peroxidase and ABTS as substrate. The synthetic peptides were P1, P2, P3, and a non-related peptide PELGASLGREYHSAR (NRP). The data are the means $\pm$ S.D. for duplicate determinations.

reactivity with the anti-NPS rabbit serum and specific reactivity with sera from *M. tuberculosis*-infected humans, were selected. The motif-containing peptides were synthesized flanked by a spacer sequence GGGS on the C-terminus, simulating the phage molecular context: peptide P1 (from clone NSP-3); P2 (from clone NSP-7) and P3 (from clone NSP-8). Binding reactivity of the synthetic peptides to the target anti-NPS antiserum was examined by ELISA using plates coated with the peptides. All the three peptides strongly bind to the antibodies (Figure 1), demonstrating that they are antigenic mimics of the NPS fraction both when they are phage displayed or chemically synthesized. To search for immunogenic properties, New Zealand rabbits were immunized with the synthetic P1, P2 or P3 oligomers. Whereas no detectable anti-NPS response could be elicited by synthetic P2 and P3, the antiserum directed against P1 showed a strong reactivity with the NPS fraction (Figure 2). This reactivity was not changed after proteinase treatment of the saccharide fraction, and was

Figure 2 Synthetic P1 is an immunogenic mimic of the NPS fraction from *M. tuberculosis*

After treatment with proteinase K, the NPS fraction was immobilized on microtitre plate wells (1 μ g sugar/well), and preimmune rabbit serum ($\circ$) or hyperimmune rabbit polyclonal antiserum raised against P1 ($\bullet$) was added at various dilutions. After developing the ELISA, the titre of the hyperimmune serum was assigned as the dilution at which a difference in absorbance A_{405} of 0.2 was observed between preimmune and hyperimmune sera (i.e. a titre of 1:400).

reduced by more than 80% after preincubation of the anti-P1 antiserum with 20 μ g of protein-free NPS fraction.

Search for sugar mimicry by synthetic peptides

The anti-NPS polyclonal antibodies used for affinity selection of phages were able to recognize purified LAM, as detected by ELISA (results not shown). This indicated that the anti-NPS antiserum recognized epitopes shared by NPS and LAM, so that the biopanning could result in the identification of peptides mimicking NPS/LAM epitopes. Within *M. tuberculosis* cell-surface NPS, antigenic components mainly include mannose-containing molecules, known to bind ConA [12]. The well-characterized mannose-containing antigens in *M. tuberculosis* are LAM [2,3], its lipid-free structurally related polysaccharide AM (arabinomannan) [7,12] and the mannosylated 45/47 kDa Apa protein [13]. We therefore observed whether the antiserum generated by immunization with the peptide mimotope P1 was capable of recognizing mannose-containing cell-envelope mycobacterial antigens.

LAM-containing *M. tuberculosis* antigenic preparations were analysed, before and after proteinase K treatment, on blots developed with anti-P1 antibodies, CS35 anti-LAM mAb or ConA. The results showed that anti-P1 rabbit antiserum strongly reacted with components showing the same electrophoretic mobility as LAM, even after the removal of protein antigens (Figure 3A). A highly purified LAM preparation was then used as antigen in an ELISA, giving definitive evidence that immunization with P1 readily elicited in the rabbit antibodies against the non-peptide lipoglycan antigen (Figure 3B). Taken together, these results proved that at least some of the identified peptides (those having the consensus sequence group I) mimic mannose-containing compounds of *M. tuberculosis*, including the major non-peptide mycobacterial antigen LAM.

Mimicked epitopes involve oligomannosidic epitopes also occurring in yeasts

Mycobacterial LAM contains lipid and saccharide immunodeterminants, the latter including both arabinosyl- and mannosyl-containing motifs [14,15]. Several biological activities of

Figure 3 The peptide P1 is an immunogenic mimic of LAM

(A) LAM-containing antigens of *M. tuberculosis* were separated by SDS/PAGE, and analysed before (–) and after (+) proteinase K treatment by silver staining and by lectin- or immunoblotting. After the treatment, a loss of protein bands was observed on silver staining (lanes 1 and 2). In contrast, proteinase-resistant reactivity of a band with electrophoretic mobility as LAM was observed in blot analyses developed with preimmune rabbit control serum (lanes 3 and 4), anti-P1 antibodies (lanes 5 and 6), CS35 mAb (lanes 7 and 8) and ConA (lanes 9 and 10). (B) LAM purified from *M. tuberculosis* cells was immobilized on microtitre plates (2 µg/well). Preimmune rabbit serum (□) or hyperimmune rabbit polyclonal antiserum raised against P1 (■) was added at various dilutions. After developing the ELISA, the titre of the hyperimmune serum was assigned as the dilution at which a difference in absorbance A_{405} of 0.2 was observed between preimmune and hyperimmune sera (i.e. a titre of 1:200).

M. tuberculosis LAM are related to the oligomannosyl caps characterizing virulent *M. tuberculosis* strains [15], and strong antigenicity is a common feature of the arabinosyl-containing mycobacterial molecules [3].

Since these saccharide capping motifs of LAM are shared by the mannosylated 45/47 kDa protein of *M. tuberculosis* [13], we looked for the reactivity of glycosylated 45/47 kDa recombinant protein expressed in *S. lividans* [6] with the hyperimmune anti-serum raised against peptide mimotope P1. As shown in Figure 4, only the ConA-binding 47 kDa Apa protein, but not the 45 kDa protein, was recognized by hyperimmune anti-P1 antiserum, when compared with serum when preimmunized control.

The anti-P1 polyclonal antibodies failed to recognize *M. tuberculosis* PIMs, which mainly include phosphatidylinositol dimannoside, and arabinofuranosyl-containing arabinogalactan (results not shown). However, a commercial preparation of *S. cerevisiae* cell-wall mannan displayed a significant capability to react with the anti-P1 antibodies (Figure 5), suggesting that the epitope mimicked by P1 (and by mimotopes having the consensus sequence group I) are oligomannosidic motifs shared by *Mycobacterium* and *S. cerevisiae* molecules.

Figure 4 Mannose-containing recombinant Apa is recognized by hyper-immune anti-P1 antiserum

The purified recombinant mannose-free 45 kDa (1) and mannose-containing 47 kDa (2) Apa proteins, expressed in *S. lividans*, were immobilized on microtitre plates (1 µg/well). Rabbit preimmune serum (hatched bars) or hyperimmune rabbit anti-P1 antiserum (filled bars) was added at dilutions of 1:100 (A) and 1:200 (B) in PBS. The assay was developed with alkaline phosphatase-conjugate and the corresponding substrate.

Figure 5 Anti-P1 antibodies show cross-reactivity with mannan from *S. cerevisiae*

A commercial preparation of *S. cerevisiae* cell-wall mannan was immobilized on microtitre plates (1.0 µg/well) and used as antigen in an ELISA with rabbit preimmune serum (□) or hyperimmune anti-P1 antiserum (■) at increasing dilutions in PBS.

DISCUSSION

Carbohydrates are crucial molecules in the interaction of pathogens with host cells. The outermost layer of *M. tuberculosis* is mainly composed of saccharides and various glycoconjugates, which seem to be involved in critical steps of tuberculosis pathogenicity [1–3]. With the purpose of identifying mimotopes of different carbohydrate epitopes representing the complex carbohydrate assemblies found in the cell-surface of *M. tuberculosis*, polyclonal antibodies directed against the outermost saccharide antigens of the bacilli were used as a target for biopanning with a combinatorial phage display peptide library. Phage clones were identified carrying dodecamer-peptides belonging to three different consensus sequences, as well as peptides with no consensus amino acid sequences (see Table 1). The recognition of both phage-displayed and synthetic peptides by the anti-carbohydrate polyclonal antiserum indicated that peptides are antigenic mimotopes resembling B-cell epitopes of *M. tuberculosis* sugars. Moreover, one of the synthetic peptides showed immunogenic properties, eliciting in rabbit antibodies directed against the NPS fraction. Remarkably, no aromatic residues were consistently present in the sequences obtained, as might be expected for peptide mimotopes of sugars [16,17]. The mechanisms of peptide-mimicking for carbohydrates are still not known. However, previous evidence suggests that the aromatic residues are critical in

peptide sequences that mimic surface conformations specifically recognized by sugar binding-ligands. A number of studies have identified carbohydrate-mimicking peptide motifs, many of them containing more than one aromatic residue [17] and it is well accepted that aromatic residues are, at least in part, responsible for the conformational mimicry of sugars [16]. Unexpectedly, the clones selected in the present study do not contain aromatic residues in the consensus motifs. Furthermore, some of the phage-displayed peptides do not contain aromatic residues at all (see Table 1). Nonetheless, functional mimicry of saccharides has also been exhibited by molecules that are very dissimilar in shape [18]. Considering that the major glycoside-containing antigens known to be present in NPS fractions of *M. tuberculosis* are mannan and AM [7,12], we investigated whether the identified peptides mimic immunodeterminants of these carbohydrate molecules. The ability of synthetic P1 to induce anti-saccharide antibodies allowed us to perform immunoassays focused to establish if the mimicked motif was present in specific carbohydrate-containing *M. tuberculosis* molecules. LAM from *M. tuberculosis* was recognized by anti-P1 antibodies, showing that the peptide mimics an epitope shared by cell-surface NPS and LAM. Although LAM has not been found in the outermost layer of a mycobacterial envelope [7], mAbs against LAM bind whole *M. tuberculosis* cells [19], as expected because the lipoglycan LAM shares glycoside epitopes with the cell-surface exposed AM. Taking into account the strong antigenic/immunogenic features of LAM and AM, it is not surprising that biopanning using polyclonal antibodies against cell-surface carbohydrates resulted in the isolation of clones carrying peptide mimotopes of these mycobacterial molecules. Other strategies should be necessary to identify the mimicked mycobacterial compound by mimotopes with other consensus sequences, since no immunogenic properties were found using synthetic linear peptides.

Whether mannosyl and/or the atypical arabinofuranosyl components of LAM are involved in the mimicked motifs is interesting, because the absence of aromatic residues in peptide mimotopes of sugars was unusual. We evaluated the reactivity of antibodies elicited by P1 against two molecules lacking arabinofuranosyl component but characterized by mannosidic epitopes present in *M. tuberculosis* LAM, namely a mannan-containing 47 kDa Apa protein, produced by recombinant methodology in *S. lividans* [6], and the cell-wall mannan from *S. cerevisiae*. The Apa protein is a 45/47 kDa *M. tuberculosis*-species specific glycoprotein complex. The protein has oligomannosidic motifs identical with the terminal mannose caps in *M. tuberculosis* LAM, which is an α (1 $\rightarrow$ 2) linked mannopyranose oligomer [13]. A recent study [6], involving one of the authors of this paper, expressed glycosylated recombinant Apa protein of *M. tuberculosis* in *S. lividans*, resulting in a 45/47 kDa doublet in which only the 47 kDa protein showed ConA-binding [6]. Although the precise glycosidic structure of recombinant glycosylated Apa is currently unknown, susceptibility to α -mannosidase and recognition by ConA of the 47 kDa protein [6] suggest that conformationally related sugars are present in both the native and recombinant Apa molecule. Similarly, *S. cerevisiae* mannan epitopes have been shown to include mainly α (1 $\rightarrow$ 2) linked mannoses [20]. Interestingly, both the mannosylated compounds were specifically recognized by antibodies elicited against peptide mimotope P1 (Figures 4 and 5). In contrast, no reactivity was found when PIMs, mainly represented by dimannosylated homologues (phosphatidylinositol dimannoside), and cell-wall arabinogalactan were used as antigens (results not shown). Altogether, these results suggest that the epitope mimicked by some of the identified peptides, specifically P1 and related peptides, are oligomannosidic epitopes also occurring in *S. cerevisiae*.

To our knowledge, this is the first report describing peptide mimotopes of *M. tuberculosis* sugars. Many important applications can be explored now with the availability of antigenic and immunogenic mimics of glycoconjugates characterizing the *M. tuberculosis* cell-envelope and potentially involved in key steps of tuberculosis pathogenicity. LAM and the related surface-exposed AM are believed to mediate mycobacterial binding and entry into host cells [1–3,21]. Both the molecules are known to elicit humoral responses, which can be exploited for tuberculosis immunodiagnosis [22,23]. LAM exhibits a broad spectrum of immunomodulatory activities [15], probably contributing to the persistence of the bacilli. Moreover, glycosyl motifs of Apa protein have been shown to be decisive in the ability of this molecule to induce T-cell responses and delayed-type hypersensitivity [24]. The preliminary results presented in this report involving human sera suggest that phage-displayed peptide mimotopes could have a potential for replacing carbohydrate antigens in immunological diagnostic tests. In this regard, important considerations about specificity should be taken into account for peptides mimicking epitopes distributed in other microorganisms. Also, it would be interesting to see how oligomannosidic epitopes mimicked by P1, which are shared by mycobacteria and *S. cerevisiae*, account for the anti-mannan reactivity characterizing sera from patients affected by Crohn's disease [20], an illness where a relationship with mycobacterial infection has been suggested. Finally, it is noteworthy that LAM and protein-carried AM have been proved to induce T-cell responses which are protective against tuberculosis, both due to CD1b-mediated responses [25] and to CD1b-independent protective mechanisms [26]. However, the immunosuppressive effects depending on LAM lipid moiety [2,15], the cumbersome nature of the methods required for their purification, and the absence of genetic strategies to obtain these kinds of molecules, may preclude the inclusion of LAM or AM in new vaccines. Taking into account the feasibility to induce T-cell responses with peptide molecules, including CD1-mediated responses [27,28], it will be very interesting to discover whether peptide mimotopes of sugars may be exploited to generate immune responses against mycobacterial non-peptide antigens, thus broadening the options for vaccine development against tuberculosis.

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REFERENCES

- Ehlers, M. R. W. and Daffé, M. (1998) Interactions between *Mycobacterium tuberculosis* and host cells: are mycobacterial sugars the key? *Trends Microbiol.* **6**, 328–335.
- Brennan, P. J. (2003) Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb.)* **83**, 91–97.
- Daffé, M. and Draper, P. (1998) The envelope layers of mycobacteria with reference to their pathogenicity. *Adv. Microb. Physiol.* **39**, 131–203.
- Scott, J. K., Loganathan, D., Easley, R. B., Gong, X. and Goldstein, I. J. (1992) A family of concanavalin A-binding peptides from a hexapeptide epitope library. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5398–5402.
- Oldenburg, K. R., Loganathan, D., Goldstein, I. J., Schultz, P. G. and Gallop, M. A. (1992) Peptide ligands for a sugar-binding protein isolated from a random peptide library. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5393–5397.
- Lara, M., Servín González, L., Singh, M., Moreno, C., Cohen, I., Nimtz, M. and Espitia, C. (2004) Expression, secretion, and glycosylation of the 45- and 47-kDa glycoprotein of *Mycobacterium tuberculosis* in *Streptomyces lividans*. *Appl. Environ. Microbiol.* **70**, 679–685.
- Lemassu, A. and Daffé, M. (1994) Structural features of the exocellular polysaccharides of *Mycobacterium tuberculosis*. *Biochem. J.* **297**, 351–357.

- 8 Sweeley, C. C., Bentley, R., Makita, M. and Wells, W. W. (1963) Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *J. Am. Chem. Soc.* **85**, 2497–2507
- 9 Gevorkian, G., Manoutcharian, K., Almagro, J. C., Govezensky, T. and Domínguez, V. (1998) Identification of autoimmune thrombocytopenic purpura-related epitopes using phage-display peptide library. *Clin. Immunol. Immunopathol.* **86**, 305–309
- 10 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Small-scale preparation of single-stranded bacteriophage M13 DNA. In *Molecular Cloning: A Laboratory Manual* (Sambrook, J., Fritsch, E. F. and Maniatis, T., eds.), pp. 4.29–4.30, Cold Spring Harbor Laboratory Press, Plainview, NY
- 11 Escamilla, L., Mancilla, R., Glender, W. and López Marín, L. M. (1996) *Mycobacterium fortuitum* glycolipids for the serodiagnosis of pulmonary tuberculosis. *Am. Rev. Resp. Crit. Care Med.* **154**, 1864–1867
- 12 Ortalo-Magné, A., Dupont, M. A., Lemassu, A., Andersen, A. B., Gounon, P. and Daffé, M. (1995) Molecular composition of the outermost capsular material of the tubercle bacillus. *Microbiology* **141**, 1609–1620
- 13 Dobos, K. M., Khoo, K. H., Swiderek, K. M., Brennan, P. J. and Belisle, J. T. (1996) Definition of the full extent of glycosylation of the 45-kilodalton glycoprotein of *Mycobacterium tuberculosis*. *J. Bacteriol.* **178**, 2498–2506
- 14 Kaur, D., Lowary, T. L., Vissa, V. D., Crick, D. C. and Brennan, P. J. (2002) Characterization of the epitope of anti-lipoarabinomannan antibodies as the terminal hexaarabinofuranosyl motif of mycobacterial arabinans. *Microbiology* **148**, 3049–3057
- 15 Nigou, J., Gilleron, M. and Puzo, G. (2003) Lipoarabinomannans: from structure to biosynthesis. *Biochimie* **85**, 153–166
- 16 Jain, D., Kaur, K., Sundaravadeivel, B. and Salunke, D. M. (2000) Structural and functional consequences of peptide-carbohydrate mimicry. Crystal structure of a carbohydrate-mimicking peptide bound to concanavalin A. *J. Biol. Chem.* **275**, 16098–16102
- 17 Monzavi-Karbassi, B., Cunto-Amesty, G., Luo, P. and Kieber-Emmons, T. (2002) Peptide mimotopes as surrogate antigens of carbohydrates in vaccine discovery. *Trends Biotechnol.* **20**, 207–214
- 18 Goel, M., Jain, D., Kaur, K. J., Kenoth, R., Maiya, B. G., Swamy, M. J. and Salunke, D. M. (2001) Functional equality in the absence of structural similarity: an added dimension to molecular mimicry. *J. Biol. Chem.* **276**, 39277–39281
- 19 Glatman-Freedman, A., Martin, J. M., Riska, P. F., Bloom, B. R. and Casadevall, A. (1996) Monoclonal antibodies to surface antigens of *Mycobacterium tuberculosis* and their use in a modified enzyme-linked immunosorbent spot assay for detection of mycobacteria. *J. Clin. Microbiol.* **34**, 2795–2802
- 20 Young, M., Davies, M. J., Bailey, D., Gradwell, M. J., Srnestad-Paulsen, B., Wold, J. K., Barnes, R. M. R. and Hounsell, E. F. (1998) Characterization of oligosaccharides from an antigenic mannan of *Saccharomyces cerevisiae*. *Glycoconj. J.* **15**, 815–822
- 21 Tailleux, L., Schwartz, O., Herrmann, J. L., Pivert, E., Jackson, M., Amara, A., Legres, L., Dreher, D., Nicod, L. P., Gluckman, J. C. et al. (2003) DC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. *J. Exp. Med.* **197**, 121–127
- 22 Navoa, J. A. D., Laal, S., Pirofski, L. A., McLean, G. R., Dai, Z., Robbins, J. B., Schneerson, R., Casadevall, A. and Glatman-Freedman, A. (2003) Specificity and diversity of antibodies to *Mycobacterium tuberculosis* arabinomannan. *Clin. Diagn. Lab. Immunol.* **10**, 88–94
- 23 Chan, E. D., Heifets, L. and Iseman, R. D. (2000) Immunologic diagnosis of tuberculosis: a review. *Tuber. Lung Dis.* **80**, 131–140
- 24 Romain, F., Horn, C., Pescher, P., Namane, A., Riviere, M., Puzo, G., Barzu, O. and Marchal, G. (1999) Deglycosylation of the 45/47-kilodalton antigen complex of *Mycobacterium tuberculosis* decreases its capacity to elicit *in vivo* or *in vitro* cellular immune responses. *Infect. Immun.* **67**, 5567–5572
- 25 Sieling, P. A., Chatterjee, D., Porcelli, S. A., Prigozy, T. I., Mazzaccaro, R. J., Soriano, T., Bloom, B. R., Brenner, M. B., Kronnenberg, M., Brennan, P. J. et al. (1995) CD-1 restricted T cell recognition of microbial lipoglycan antigens. *Science* **269**, 227–230
- 26 Hamasur, B., Haile, M., Pawlowski, A., Schröder, U., Williams, A., Hatch, G., Hall, G., Marsh, P., Källénius, G. and Svenson, S. B. (2003) *Mycobacterium tuberculosis* arabinomannan-protein conjugates protect against tuberculosis. *Vaccine* **21**, 4081–4093
- 27 Castaño, A. R., Tangri, S., Miller, J. E., Holcombe, H. R., Jackson, M. R., Huse, W. D., Kronenberg, M. and Peterson, P. A. (1995) Peptide binding and presentation by mouse CD1. *Science* **269**, 223–226
- 28 Moody, D. B., Young, D. C., Cheng, T. Y., Rosat, J. P., Roura-Mir, C., O'Connor, P. B., Zajonc, D. M., Walz, A., Miller, M. J., Levery, S. B. et al. (2004) T cell activation by lipopeptide antigens. *Science* **303**, 527–531

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