

Specific and Randomly Derived Immunoactive Peptide Mimotopes of Mycobacterial Antigens[∇]

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The mycobacterial cell surface contains complex nonprotein antigens that are highly immunoactive in nature. However, these antigens are chemically heterogeneous and structurally complex, thereby limiting their applications. To identify their peptide mimotopes, phage-displayed peptide libraries Ph.D.-7 and Ph.D.-12 were panned on either defined template, monoclonal antibody (MAb) CS-35 against lipoarabinomannan (LAM), or a polyclonal rabbit immune serum reactive against whole cells of *Mycobacterium bovis* BCG. Panning on anti-LAM MAb CS-35 yielded two confirmed mimotopes of LAM, a 7-mer and a 12-mer, whereas panning on polyclonal serum yielded a large repertoire of mimotopes reactive against sera from BCG-immunized rabbits, one of which turned out to have the same sequence as the 7-mer LAM mimotope. The dissociation constant of the interaction between MAb CS-35 and a synthetic peptide corresponding to the 7-mer LAM mimotope was determined to be 7.55 μ M. Dot blot assays were performed with peptides corresponding to the two LAM mimotopes to evaluate their diagnostic potential. Both peptides gave discernibly higher signals with a panel of tuberculosis (TB) patient sera than with sera from healthy controls. The peptides were also found to stimulate the release of tumor necrosis factor alpha and interleukin-12 cytokines in the J774A.1 cell line and primary bone marrow-derived macrophages, indicating that they may have immunomodulatory potential. The present study demonstrates that peptide mimotopes of known and unknown mycobacterial antigens could be isolated by using subtractive phage display techniques and that these peptides could have potential applications in areas such as TB diagnostics and immunotherapy.

The mycobacterial cell wall is rich in immunoactive macromolecules (7, 8), many of which are involved in the pathology of tuberculosis (TB) (3), a disease that has killed millions in the past and that continues to do so at present (35, 59). TB has been studied not only because of its medical importance but also because it is considered a very interesting system that can be used to provide an understanding of the host-pathogen relationship. In particular, TB is a useful model with which to study how pathogens evade host responses. Although the phenomenon is complex, many pieces of evidence suggest that mycobacteria can manipulate host responses, usually to their own benefit (38). As in the case of other pathogens, the first encounter between mycobacteria and the host cells is through surface-to-surface contact. This involves different types of receptors on the part of the host cells and a variety of ligands that are exposed on the surface of the mycobacteria. Many of these ligands are complex sugars, and they function to activate various cellular responses in the host (4, 16).

The immune system reacts to mycobacterial invasion through both T-cell and B-cell responses (48). Many antigens which activate both these wings of the immune system have been discovered. A large number of these antigens are proteins (36, 43, 44, 49); their genes have been cloned and expressed, and minute details of their T- or B-cell epitopes have been characterized. Several of these do find application as diagnostic tools (11, 17, 47, 58) and subunit vaccines (1, 6, 14, 23). The

nonprotein antigens, however, are equally important in the antigenic responses, and many of them, such as lipoarabinomannan (LAM), phenolic glycolipids, and cord factor, have been used as diagnostic agents (21, 26, 27, 29, 54). Unlike protein antigens, however, which can be cloned and expressed at will, the nonprotein antigens cannot be studied through cloning techniques. Hence, these antigens must be purified from mycobacterial whole cells by complex procedures.

The advent of the phage display technology has ushered in the ability to raise peptide mimotopes of protein as well as nonprotein antigens. The ability to derive peptide mimotopes has made it possible to use them in diagnostic assays in place of various antigens (30), particularly nonprotein antigens, such as lipopolysaccharides (LPSs) (12). The phage display technique also has an added advantage: it can be used even when the nature of the antigen is not known (50). This is particularly important, since many antigenic determinants may not exist as discrete entities and could be “conformational” in nature; i.e., a certain pattern rather than a definite structure could play the role of an antigenic determinant or immunomodulator. In some cases, such as cancer, rheumatoid arthritis and AIDS, in which the precise antigen or immunogenic epitopes were not known, differential phage display library screenings have been done with sera from patients and healthy individuals (15, 33, 46).

Given the remarkable fidelity of the phage display technique, it was decided to apply this technique to raise peptide mimotopes of mycobacterial cell surface antigens. The immediate objective was to explore the possibility of obtaining markers for the serodiagnosis of TB, but in addition, the possibility of using such peptide mimotopes as immunomodulatory agents was also looked into. Two approaches were taken, one of

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which was to use a well-characterized monoclonal antibody (MAb) against an immunodominant mycobacterial cell surface antigen, LAM, and the other was to use polyclonal serum of rabbit immunized with whole *Mycobacterium bovis* BCG cells as the template for the screening of phage-displayed peptide libraries. The two approaches led us to identify mimotopes that could be potentially useful as diagnostic agents. In addition, it could be demonstrated, in principle, that the mimotopes raised by such methods could function as immunomodulators as well.

MATERIALS AND METHODS

Phage display libraries. Rationally designed combinatorial phage display libraries of peptide sequences (7-mer or 12-mer), inserted into the NH₂ terminus of the pIII minor coat protein of the M13 bacteriophage, were obtained from New England Biolabs, Inc., Beverly, MA. These libraries (termed Ph.D.-7 and Ph.D.-12) contained either 7 amino acids (for the Ph.D.-7 library) or 12 amino acids (for the Ph.D.-12 library). The peptides were followed by a short spacer (Gly-Gly-Gly-Ser) and then the wild-type pIII sequence. The Ph.D.-7 library consisted of 2.8×10^9 independent clones, sufficient to encode most, if not all, of the 20⁷, or 1.28×10^9 , possible 7-residue sequences; and the Ph.D.-12 library consisted of 1.9×10^9 independent clones, sufficient to encode 20¹², or 4.1×10^{15} , possible 12-residue sequences. In these libraries 20 amino acids were coded by only 32 codons, as a result of which the relative frequency of residues with a single codon increased.

Bacterial strains and growth conditions. The *Mycobacterium bovis* BCG strain was a generous gift from Anil Tyagi, Department of Biochemistry, University of Delhi (South Campus), New Delhi, India. It was grown in Middlebrook 7H9 broth (Difco, Becton Dickinson and Co. [BD]) supplemented with 10% oleate-albumin-dextrose-catalase enrichment (Difco, BD, USA), 0.5% glycerol, and 0.2% Tween 80 until mid-log phase at 37°C with continuous shaking. *Mycobacterium smegmatis* LR222 was grown at 37°C under shaking conditions in 2× yeast extract-tryptone medium supplemented with 0.5% glycerol and 0.2% Tween 80 for 3 days. *Escherichia coli* ER2738 was obtained from New England Biolabs, Inc. It was grown in Luria-Bertani broth containing 12.5 µg tetracycline/ml at 37°C with continuous shaking.

Murine macrophage culture. The murine macrophage-like cell line J774A.1 was obtained from the National Centre for Cell Science (Pune, India). Murine bone marrow-derived macrophages (BMMφ) were generated from bone marrow stem cells obtained from femur and tibial washouts of BALB/c mice, which were obtained from National Centre for Laboratory Animal Sciences (Hyderabad, India). Three mice (ages, 6 to 8 weeks), irrespective of their sex, were used for each experiment. The cell line was maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (HyClone Laboratories Inc., South Logan, Utah), 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated in a 37°C chamber with 5% CO₂ and 95% humidity. Murine bone marrow stem cells were cultured in the medium described above supplemented with 10% L-929 cell-conditioned medium as a source of macrophage colony-stimulating factor (9, 41) for 5 to 6 days in a humidified chamber at 37°C with 5% CO₂ to obtain differentiated BMMφ, which were maintained in the same medium.

Anti-LAM MAb. An immunoglobulin G3 (IgG3) anti-LAM mouse MAb, MAb CS-35, was acquired as a tissue culture supernatant in RPMI medium containing 10% fetal calf serum from Patrick J. Brennan through Leprosy Research Support, National Institutes of Health (NIH), National Institute of Allergy and Infectious Diseases (NIAID) (contract NO1-AI-25469), Colorado State University, Fort Collins, CO. When necessary, the IgG fraction was purified by using protein A-Sepharose beads (Amersham Biosciences, Uppsala, Sweden) from the supplied tissue culture supernatant by diluting it 1:1 in 1.5 M glycine-NaOH buffer (pH 9) containing 3 M NaCl. Protein A-Sepharose beads were equilibrated with the same buffer, and binding with the diluted tissue culture supernatant was allowed to proceed for 30 min at room temperature and then for 1 h on ice. The beads were washed 10 times to remove unbound contaminants, and the bound antibody was eluted with 0.2 M glycine-HCl buffer (pH 2.2) in 0.5-ml fractions, which were then neutralized with 75 µl of 1 M Tris-HCl (pH 9.1). The fractions were analyzed and were found to be >98% pure by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The eluted fractions containing antibody were pooled and dialyzed overnight at 4°C against 2 liters of phosphate-buffered saline (PBS; 15 mM sodium phosphate buffer [pH 7.4] containing 150 mM NaCl). The antibody concentration was determined by using the method of Lowry et al. (31).

Lipoarabinomannan antigens. Two different types of lipoarabinomannan antigens were used in this study. Mannose-capped LAM (ManLAM) from *Mycobacterium tuberculosis* H37Rv was kindly provided by John Belisle through TB Research Materials & Vaccine Testing, NIH, NIAID (contract NO1-AI-75320), Colorado State University. Phosphoinositol-capped LAM (PILAM) from *M. smegmatis* LR222 was purified according to the rapid large-scale LAM purification method of Hamasur et al., which included gel-exclusion chromatography on a Sephacryl S-100 column (Amersham Pharmacia Biotech, Uppsala, Sweden) for further purification (20). The concentration of PILAM was estimated from immunoblots by using MAb CS-35 and by taking the concentration of *M. tuberculosis* H37Rv LAM supplied by the TB Research Materials & Vaccine Testing contract as a standard. Both ManLAM and PILAM contained <10-ng/mg endotoxin contamination, as determined by the *Limulus* amoebocyte assay.

Anti-BCG rabbit sera. Antisera were raised against heat-killed *M. bovis* BCG cells in three rabbits individually. The *M. bovis* BCG culture was grown as described above, and the cells were harvested by centrifugation at $4,000 \times g$ for 20 min. The cell pellet was washed twice with PBS and was resuspended in PBS at an optical density (OD) at 600 nm (OD₆₀₀) of 1. This suspension was heat killed at 100°C for a maximum of 10 min. The suspension obtained as described above was centrifuged at $10,000 \times g$ for 10 min, and the pellet was resuspended in PBS. This bacterial suspension was diluted for two subcutaneous (s.c.) injections (2×10^7 cells/ml) and four intravenous (i.v.) injections (increasing doses of 2×10^7 cells/ml, 4×10^7 cells/ml, 8×10^7 cells/ml, and 1.6×10^8 cells/ml) to be given to three individual rabbits and stored in aliquots at -70°C until it was required. There was an interval of 2 weeks between the first and the second s.c. injections and between the second s.c. injection and the first i.v. injection and a 5-day interval between subsequent i.v. injections. A preimmune bleeding was done before immunization, and the final bleeding was done after the fourth i.v. immunization. The sera were separated according to the standard protocol and were stored in aliquots at -20°C until they were used.

Human sera. Blood was collected by vein puncture from five patients under medication from a nearby hospital (Employee State Insurance Hospital, Kolkata, India) who were diagnosed with TB (acid-fast smear positive or culture positive). Blood was also drawn from five healthy individuals (research scholars of the Bose Institute, Kolkata, India) who had been vaccinated with BCG during childhood. Sera were separated according to the standard protocol and were stored in aliquots at -20°C until they were used.

SDS-PAGE and Western blotting of *M. tuberculosis* H37Rv LAM with antisera raised against *M. bovis* BCG cells. H37Rv LAM (1 µg/well) was electrophoresed in a 15% separating and a 6% stacking SDS-polyacrylamide gel at a constant current of 20 mA for approximately 90 min and transblotted to Immobilon-PS^Q polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA). Western blotting with rabbit preimmune and immune sera was done according to the standard protocol by using a commercial kit (Boehringer Mannheim chromogenic Western blotting kit; Roche, Mannheim, Germany). The membranes were developed with *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) ready-to-use substrate solution (provided with the same kit).

Screening of phage display libraries. Phage display libraries Ph.D.-7 and Ph.D.-12 were screened by biopanning in solution with protein A-Sepharose beads to affinity capture the antibody-phage complex, according to standard methods, with a few modifications. Briefly, 20 µl of protein A-Sepharose beads (50% aqueous suspension) was washed with Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.5] containing 150 mM NaCl) containing 0.1% (vol/vol) Tween 20 (TBST) and blocked with 0.1 M NaHCO₃ buffer (pH 8.6) containing 5 mg of bovine serum albumin (BSA)/ml for 1 to 2 h at 4°C with occasional mixing. A total of 10¹¹ PFU of the Ph.D.-7 or Ph.D.-12 phage library was incubated with 10 nM (final concentration) MAb CS-35 in 200 µl TBST for 20 min at room temperature. The blocked protein A-Sepharose beads were washed three to four times with TBST, and the phage-antibody mixture was incubated with the washed beads for 15 min at room temperature with occasional mixing. After unbound phage was removed by washing 10 times with TBST, the phage bound to MAb CS-35 was eluted with 0.2 M glycine-HCl buffer (pH 2.2) containing 1 mg/ml BSA. After elution the acidic buffer was neutralized with 1 M Tris-HCl (pH 9.1). The eluted phage was amplified in vivo in host strain *E. coli* ER2738, purified by double precipitation in the cold with 1/6 volume of polyethylene glycol (PEG)-NaCl (20% [vol/vol] polyethylene glycol 8000, 2.5 M NaCl), and used in a second round of biopanning. The panning and elution processes were carried out three times. The stringency of selection was gradually increased with each round by raising the Tween 20 concentrations in wash buffer stepwise from 0.1% to 0.3% and then to 0.5% in the three rounds of panning. A subtractive panning step with protein A-Sepharose beads was included in the second and third rounds. For subtraction, the phage pool obtained from a particular panning step was reacted

with blocked protein A-Sepharose beads. The beads along with the bound phage were discarded, and the supernatant was used for the next panning step. A similar protocol was followed for the screening of the Ph.D.-7 library against anti-BCG rabbit polyclonal serum, except that 1 μ l of immune serum was used instead of MAb. In this case three rounds of panning were done, with the protein A-Sepharose subtraction step included as described above. An additional subtractive panning step was included after the third round by using preimmune serum to eliminate background binding. For this, the phage pool from the third round was incubated with 1 μ l of preimmune serum, and the antibody-phage complex was captured on the beads and discarded. After three rounds of positive selection, the *E. coli* ER2738 cells were infected with the eluted phage and grown on Luria-Bertani agar plates coated with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and IPTG (isopropyl-1-thio- β -D-galactopyranoside) (Sisco Research Laboratory, Mumbai, India). Individual plaques were picked at random and amplified, and single-stranded phage DNA was isolated for sequencing. The sequencing was done with the -96 gIII sequencing primer (New England Biolabs, Inc.) and dye-labeled dideoxynucleotides on an automated cycle sequencer (Applied Biosystems) at the Bangalore Genei Sequencing Facility, Peenya, Bangalore, India. The amino acid sequences of the peptide inserts were analyzed by a computer search with the ExPASy tools server (<http://www.expasy.ch/tools>). Consensus sequences were determined by multiple-sequence alignment with the T-Coffee program.

Reverse phage ELISA. A reverse phage enzyme-linked immunosorbent assay (ELISA) was used to evaluate the ability of selected phage clones to bind to the MAb CS-35 and anti-BCG polyclonal sera. Briefly, either 200 μ l/well of the MAb tissue culture supernatant, a 1:40 dilution of the rabbit serum, or 1 μ g/well of the purified antibody in 0.05 M Na₂CO₃-NaHCO₃ buffer (pH 9.6) was added to the required number of wells of a 96-well Maxisorp microtiter plate (Nunc, Roskilde, Denmark) and blocked with 3% nonfat milk powder in TBS (Bangalore Genei Pvt. Ltd., Peenya, Bangalore, India). The unrelated MAb CS-49, which is reactive against HspX (Rv2031c) of *M. tuberculosis*, and rabbit preimmune sera were used as the respective negative controls. The unselected Ph.D.-7 or Ph.D.-12 phage library (as the negative control) and the selected peptide phage clones, which had been amplified and concentrated by the PEG-NaCl double-precipitation method, were added to each coated well ($\sim 10^9$ to 10^{10} PFU/well), and the plates were incubated for 2 h at room temperature. Unbound phage was removed by washing with TBST (0.5% [vol/vol] Tween 20), and bound phage was detected with horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal antibody (1:5,000) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate (Roche, Mannheim, Germany). The OD at 405 nm was determined with a microplate reader (model 680; Bio-Rad Laboratories, Richmond, CA).

ELISA for identification of antibody against LAM. ELISA was used to evaluate human sera for the presence of antibody against LAM (22). Briefly, 96-well Maxisorp microtiter plates (Nunc) were coated overnight with *M. tuberculosis* H37Rv LAM (1 μ g/well) diluted in 0.05 M Na₂CO₃-NaHCO₃ buffer (pH 9.6) and blocked with 5 mg/ml BSA and 1% gelatin in PBS at 37°C for 2 h. The test sera, diluted 1:50 in dilution buffer (0.2% BSA, 0.05% gelatin, 0.2% Tween 20 in PBS), were incubated for 1 to 2 h at 37°C. Negative controls were wells without test sera but with dilution buffer only. After the washing of unbound antibody with PBST (0.05% Tween 20 in PBS), bound antibody was detected with 1:10,000-diluted HRP-conjugated goat anti-human IgG (Sigma Immunochemicals, St. Louis, MO) and *o*-phenylenediamine (OPD; Sigma Chemical Co., St. Louis, MO) substrate. The OD at 492 nm was determined with a microplate reader (Anthos Labtec Instruments, Salzburg, Austria).

Peptide synthesis. For the derivation of synthetic peptides corresponding to the selected LAM mimotopes, a structurally flexible linker (Gly-Gly-Gly-Ser) was added to the representative consensus sequences at the C-terminal ends to obtain the effective conformations of peptides. Peptides were produced on a 0.12-mmol scale by using standard 9-fluorenylmethoxy carbonyl chemistry, as described earlier (45). The purity and identity of the peptides were confirmed by time of flight-electrospray mass spectrometry on a Micromass Q-T spectrometer (Waters, Milford, MA). The concentrations of the peptides were determined by the method of Lowry et al. (31).

Determination of binding by fluorescence enhancement. The dissociation constant (K_D) for peptide binding to the MAb CS-35 was determined essentially as described earlier (25, 39). The assay is based on the increase in the tryptophanyl fluorescence of the MAbs following the interaction with antigens. A synthetic peptide corresponding to a LAM mimotope was used as the antigen. Only the binding of those peptides that lack intrinsic tryptophanyl fluorescence can be assessed by this method. Fluorescence measurements were taken in a Hitachi F-3010 spectrofluorimeter, which has a facility for spectrum addition and subtraction. The excitation and emission wavelengths were 295 nm and 333 nm,

respectively. The excitation and emission band passes were 10 nm and 20 nm, respectively. Purified MAb CS-35 (0.3 μ M) was titrated with increasing concentrations of PepCS-35[7] in 200 μ l of PBS at room temperature ($25 \pm 1^\circ$ C). The observed fluorescence values were corrected for the small volume changes that occurred during the titration. The inner filter effect was negligible. For each datum three measurements were taken, and the fluorescence values, which did not vary by more than 0.5%, were averaged. F/F_0 (where F_0 is the initial fluorescence and F is the fluorescence obtained at a particular concentration of peptide) values were determined. Three experiments were performed, and the mean F/F_0 values \pm the standard errors of the means (SEMs) were plotted against the peptide concentration. The F/F_0 data were fitted to a single-step binding equation (equation 1) by using the Marquardt nonlinear least-squares algorithm to determine the apparent dissociation constant of the peptide-antibody complex (45).

$$Q = 1 + (Q_\alpha - 1) \cdot \{(\text{Pep}_{\text{tot}} + \text{Pro}_{\text{tot}} + K_D) - \sqrt{[(\text{Pep}_{\text{tot}} + \text{Pro}_{\text{tot}} + K_D)^2 - (4 \cdot \text{Pep}_{\text{tot}} \cdot \text{Pro}_{\text{tot}})]}\} / (2 \cdot \text{Pro}_{\text{tot}}) \quad (1)$$

where Q is the observed fluorescence ratio, Q_α is the maximum peptide-induced fluorescence ratio of the antibody, Pep_{tot} is the total concentration of the peptide, and Pro_{tot} is the total concentration of the protein. The parameters of the fit were Q_α and K_D . Curve fitting and statistical deductions were done by using Kyplot software (K. Yoshioka, 1997 to 1999, version 2.0 beta 4).

Dot blot analysis of LAM peptide mimotopes. A dot blot technique was used to analyze the binding of anti-LAM antibody to LAM and synthetic peptides. *M. tuberculosis* H37Rv LAM (0.1 μ g per well) or synthetic peptides (5 μ g per well) were immobilized onto a pretreated Immobilon-P^{SO} PVDF membrane (Millipore Corporation) by using a minifold dot blot apparatus (Schleicher & Schuell, Inc., Keene, NH), according to the manufacturer's recommendations. The membrane was then removed and blocked with 3% nonfat milk powder in TBS at 37°C for 2 h. The blocked membranes were then incubated for 2 h at room temperature and then overnight at 4°C with rabbit sera (preimmune or immune) and human sera (healthy control sera and TB patient sera) at 1:500 and 1:100 dilutions, respectively, in blocking solution. Antibody bound to the membranes was detected with anti-rabbit IgG-anti-mouse IgG alkaline phosphatase (AP) conjugate (the same conjugate used for Western blotting) or 1:3,000-diluted rabbit anti-human IgG AP conjugate (Bangalore Genei Pvt. Ltd.) and NBT-BCIP substrate, as mentioned above for Western blotting. Spot intensities were determined by densitometric scanning of the blots by using a GS-700 imaging densitometer (Bio-Rad Laboratories) and analyzed by the Molecular Analyst (version 1.5) software supplied with the densitometer.

Cytokine ELISA. Wells of flat-bottom 24-well tissue culture plates (Costar, Cambridge, Mass) were seeded with 2×10^5 to 3×10^5 J774A.1 cells or BMM ϕ in a total volume of 500 μ l per well (two replicates per treatment). The cells were incubated with medium alone (no stimulation) or medium plus various modulators (PILAM, ManLAM, LPS, or synthetic peptides corresponding to LAM mimotopes) at the desired concentrations for 24 h at 37°C in a humidified chamber with 5% CO₂. Stimulation with LPS from *E. coli* (Sigma Chemical Co.) was done as a positive control. The cell culture supernatants were harvested from each well after 24 h. The supernatants were either analyzed immediately or stored at -70°C until further use. A sandwich ELISA for the detection of interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF- α) was performed with the same lot of supernatant by using matched monoclonal antibody pairs against the respective cytokines (BD Pharmingen, San Diego, CA), and detection was done by using streptavidin-HRP as the enzyme reagent and OPD as the substrate. The OD at 492 nm was determined. The absence of cytotoxicity of the stimuli was monitored by performing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide conversion assay. The cell viability was found to be $\geq 95\%$ by the use of all the treatments described above.

Statistical analysis. Replicate experiments were done thrice independently, and summary results are represented as means \pm standard deviations (SDs) or SEMs, as may be the case. Kyplot software (K. Yoshioka, 1997 to 1999, version 2.0 beta 4) was used for all statistical deductions. Group means were compared by Student's *t* test for paired comparisons. The significance levels were indicated, wherever relevant, by the *P* values. Differences were considered significant for *P* values of <0.05.

RESULTS

Biopanning on anti-LAM MAb CS-35 for screening of LAM mimotopes. Anti-LAM MAb CS-35 was used for the direct isolation of mimotopes of LAM, which is one of the prominent

TABLE 1. MAb CS-35 binding peptide sequences selected after third round of panning

Clone sequence identifier	Amino acid sequence	Frequency ^a
CS-35[7]-1 to -6	Met-Ser-Pro-Arg-Ala-Thr-Ile	6/10
CS-35[7]-7	Lys-Leu-Met-Thr-His-Trp-Pro	1/10
CS-35[7]-8	Gly-Leu-Ser-Leu-Pro-Pro-Gly	1/10
CS-35[7]-9	Leu-Pro-Asp-Thr-Leu-Ser-Ser	1/10
CS-35[7]-10	Gln-Pro-Pro-Leu-Thr-Leu-Asn	1/10
CS-35[12]-1 to -8	Ser-His-Arg-Leu-Leu-Gln-Thr-Tyr-Trp-Ser-Ser-Ala	8/9
CS-35[12]-9	Tyr-Met-Asp-Thr-Gln-Thr-Thr-Leu-Pro-Ile-Met-Trp	1/9

^a Number of clones with the sequence/total number of clones tested.

immunoactive antigens on the mycobacterial cell surface. After the third round of panning, the peptide-coding regions of randomly selected phage clones were sequenced. The results (Table 1) showed that in the case of both libraries, Ph.D.-7 and Ph.D.-12, recurring sequences could be detected, indicating their preferential binding with the target. In the case of the Ph.D.-7 library, 6 of the 10 randomly selected phage clones belonged to a group (CS-35[7]-1 to CS-35[7]-6), in which all the clones had the same sequence (Met-Ser-Pro-Arg-Ala-Thr-Ile). Similarly, in the case of the Ph.D.-12 library, eight of the nine randomly selected phage clones (CS-35[12]-1 to CS-35[12]-8) could be grouped together, as all of them had the sequence Ser-His-Arg-Leu-Leu-Gln-Thr-Tyr-Trp-Ser-Ser-Ala. One member from each group, CS-35[7]-1 and CS-35[12]-2, respectively, was arbitrarily selected for further confirmation of their binding to MAb CS-35 by reverse phage ELISA.

Binding affinity of the selected phage clones for MAb CS-35.

A reverse phage ELISA was done with MAb CS-35-coated microtiter plates. Unrelated MAb CS-49 was used as a negative control. Both the MAb tissue culture supernatant (Fig. 1A) and the protein A-Sepharose-purified MAb (Fig. 1B) were used in the assay. Figure 1A and B show a comparative evaluation of the reactivity of the selected phage clones (CS-35[7]-1 and CS-35[12]-2) and the unselected Ph.D.-12 library toward the two MAbs. Both clones reacted with CS-35 but not CS-49. Furthermore, the unselected Ph.D.-12 library did not react to any significant extent with either MAb. Hence, the CS-35[7]-1 and CS-35[12]-2 peptide phage clones bind to MAb CS-35 specifically. However, the reactivity of CS-35[12]-2 was higher than that of CS-35[7]-1. The results were same irrespective of whether the tissue culture supernatant or the purified antibody was used.

Enrichment of anti-BCG rabbit immune serum-specific phage-displayed peptide sequences through biopanning.

Three rabbits were similarly immunized with heat-killed whole *M. bovis* BCG cells, and a panel of sera which should contain polyclonal antibodies that mainly recognize surface-exposed mycobacterial antigens was thus raised. These sera were arbitrarily designated R1, R2, and R3. To confirm that the immunization did result in a response against mycobacterial surface antigens, Western blotting was carried out against LAM, which is well known as an important surface antigen, by using the R1, R2, and R3 rabbit sera. The results of the Western blotting (Fig. 2) indicated that the three immune sera showed strong

cross-reactivity with LAM at dilutions of 1:1,000, whereas the corresponding preimmune sera showed no cross-reactivity even at a 1:100 dilution.

Biopanning of the Ph.D.-7 library was performed with R1 rabbit serum. Three rounds of panning were performed with a subtraction step with R1 preimmune serum, which was included after the third panning. The degree of enrichment was pursued by performing reverse phage ELISA with R1, R2, and R3 preimmune and immune sera. The results (Fig. 3) showed that there was a substantial enrichment of R1 immune serum binding phages relative to that of R1 preimmune serum binding phages at each round of panning. As expected, the R1 preimmune serum subtraction step resulted in a further increase in the enrichment for R1 immune serum binding phages. Also, the final enriched phage pool showed significant ($P < 0.001$) preferential binding with R2 and R3 immune sera

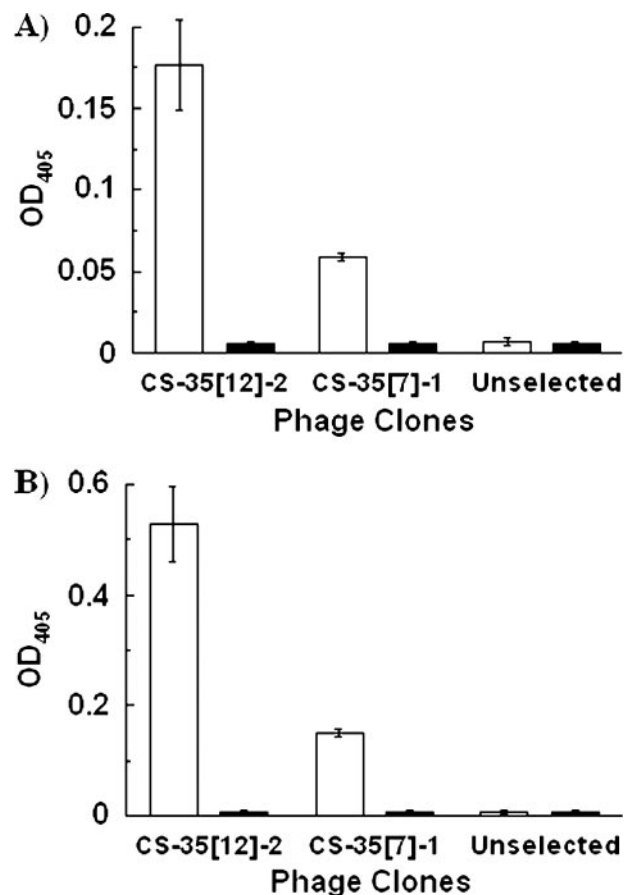


FIG. 1. Reverse phage ELISA to confirm the binding of selected phage clones to MAb CS-35. The phage clones CS-35[7]-1 and CS-35[12]-2 and the unselected Ph.D.-12 library were amplified; concentrated through double PEG precipitation; and reacted with MAb CS-35 (white bars) and unrelated MAb CS-49 (black bars), which were coated on the wells of a 96-well microtiter plate. Results are presented for (A) the MAb tissue culture supernatant (200 μ l per well) and (B) the protein A-Sepharose-purified MAb (1 μ g per well). Unbound phage was removed by washing with TBST (0.5% [vol/vol] Tween 20), and bound phage was detected with HRP-conjugated anti-M13 monoclonal antibody (1:5,000) and ABTS substrate. Color development was monitored at 405 nm. The results are expressed as the means \pm SDs for three independent experiments.

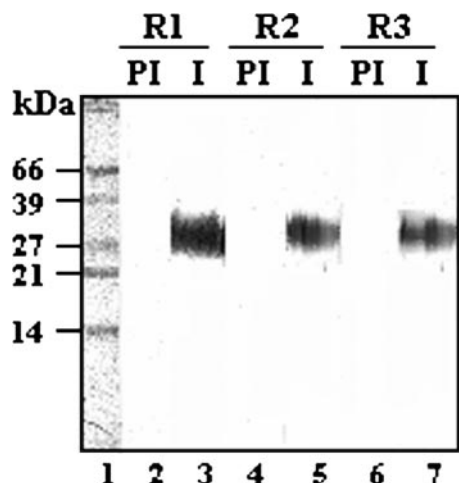


FIG. 2. Western blot analysis of LAM with antisera raised against *M. bovis* BCG cells in three individual rabbits. Antisera against heat-killed *M. bovis* BCG cells were raised in three rabbits individually by using a similar immunization protocol. These sera were arbitrarily designated R1, R2, and R3. ManLAM from *M. tuberculosis* H37Rv (1 µg/well) was subjected to SDS-PAGE (15%) and transblotted onto a PVDF membrane. Immunoblotting was done with the three rabbit preimmune sera (PI; 1:100 dilution) and immune sera (I; 1:1,000 dilution). Lanes: 1, molecular mass marker (Coomassie blue stained); 2, R1 preimmune serum; 3, R1 immune serum; 4, R2 preimmune serum; 5, R2 immune serum; 6, R3 preimmune serum; 7, R3 immune serum.

relative to that with the corresponding preimmune sera. The difference in binding specificities for the three rabbit immune sera was related to the enrichment process, as the unselected Ph.D.-7 library showed no preferential binding with any of the

TABLE 2. R1 immune serum binding peptide sequences selected after third round of panning with the Ph.D.-7 library

Clone sequence identifier	Amino acid sequence	Frequency ^a
R1[7]-1 to -4	Met-Ser-Pro-Arg-Ala-Thr-Ile	4/30
R1[7]-5 to -10	Glu-Gln-Pro-Tyr-Leu-His-Val	6/30
R1[7]-11 and -12	Glu-Gln-Pro-Tyr-Ile-Glu-Asn	2/30
R1[7]-13	Glu-Gln-Pro-Tyr-Arg-Ser-Met	1/30
R1[7]-14 and -15	Ser-Met-Ile-Thr-Asp-Leu-Leu	2/30
R1[7]-16	Ser-Met-Ile-Arg-Asp-Leu-Leu	1/30
R1[7]-17 and -18	Ser-Met-Met-Thr-Glu-Leu-Leu	2/30
R1[7]-19 and -20	Met-Pro-Phe-Val-Thr-His-Asn	2/30
R1[7]-21	Asn-Leu-Thr-Asp-Ile-Asn-Leu	1/30
R1[7]-22	Asn-Leu-Thr-Asp-Ile-Leu-Pro	1/30
R1[7]-23	Thr-Met-Asp-Leu-Gly-Arg-Phe	1/30
R1[7]-24	Tyr-Met-Asp-Leu-Gly-Met-Lys	1/30
R1[7]-25	Leu-Ser-Gln-Asn-Ala-Ser-Val	1/30
R1[7]-26	Pro-Ala-Pro-Asn-Ala-Ser-Leu	1/30
R1[7]-27	Ala-Val-Gln-Gly-Phe-Asn-Trp	1/30
R1[7]-28	Ala-Thr-His-Phe-Met-Arg-Ile	1/30
R1[7]-29	Lys-Pro-Ser-Asp-Phe-Pro-Pro	1/30
R1[7]-30	Gln-Pro-Leu-His-Ser-Pro-Leu	1/30

^a Number of clones with the sequence/total number of clones tested.

three immune sera relative to that with the corresponding three preimmune serum samples.

Thirty individual plaques from the third panning were selected at random, and the peptide-coding region was sequenced. The clones with similar sequences were grouped and numbered essentially as done in the case of panning against the monoclonal antibody. The results (Table 2) showed that one of the groups represented by R1[7]-1 had the same sequence as the group represented by CS-35[7]-1. Thus, although two in-

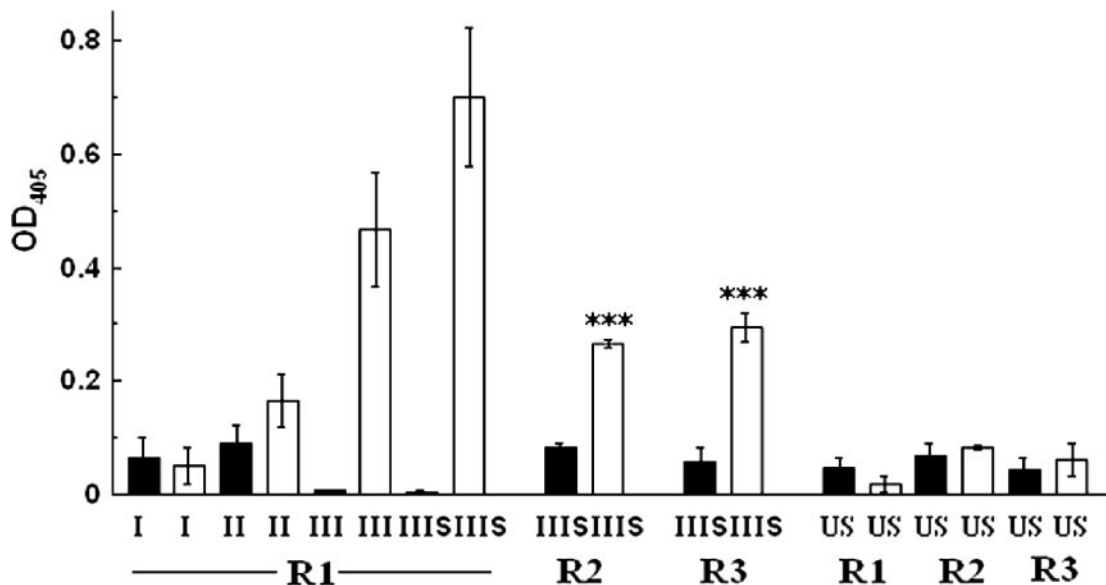


FIG. 3. Reverse phage ELISA to pursue the degree of enrichment of immune serum-specific phage pool. Phage pools obtained after each round of panning of the Ph.D.-7 library (I, II, III, and IIIS [III after R1 preimmune serum subtraction]) against R1 immune serum were subjected to reverse phage ELISA with R1 immune serum (white bars) and the corresponding preimmune serum (black bars) coated at a 1:40 dilution on a 96-well microtiter plate. The phage pool from the IIIS step was also assayed with R2 and R3 immune and preimmune sera (the bars are the same as those for the R1 serum). To demonstrate the specificity of the reactivity of enriched pools, a control experiment was performed with unselected Ph.D.-7 library (US). The results are expressed as the means ± SDs for three independent experiments. ***, a significance level of $P < 0.001$ relative to the results for the corresponding preimmune sera.

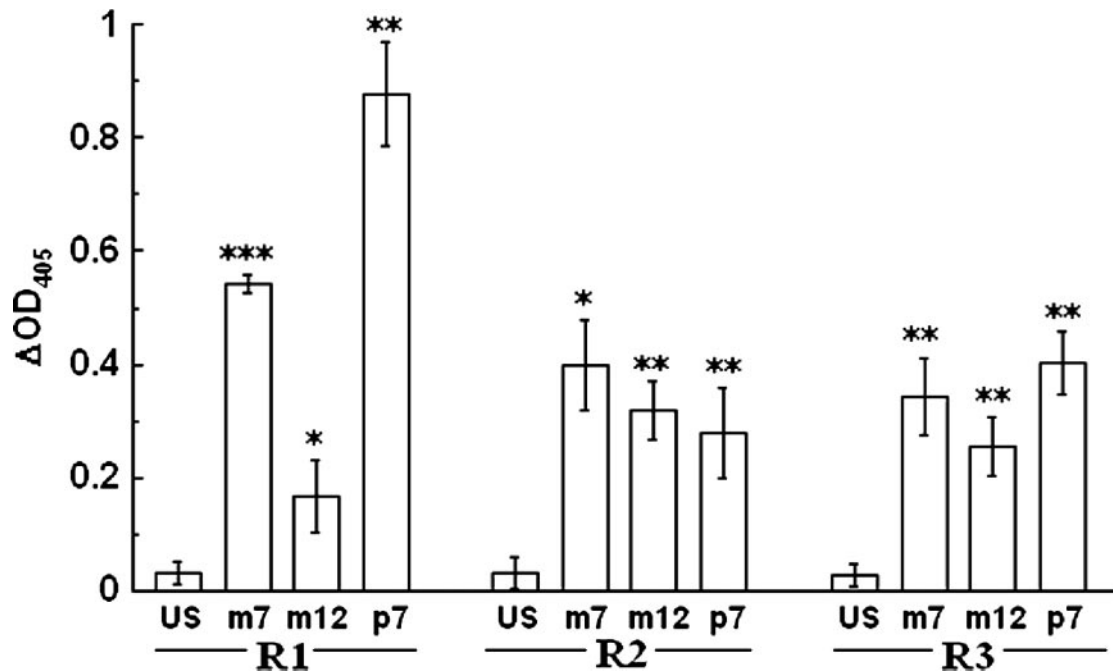


FIG. 4. Preferential binding of selected individual phage clones to rabbit anti-BCG polyclonal sera. Reverse phage ELISA was performed with phage clones CS-35[7]-1, CS-35[12]-2, and R1[7]-5; the unselected Ph.D.-7 library; and the three preimmune and immune rabbit sera (R1, R2, and R3), coated at a 1:40 dilution on a 96-well microtiter plate. The unselected Ph.D.-7 library was used as the negative control. Bars represent the ΔOD_{405} at 405 nm for the immune sera and the preimmune sera for CS-35[7]-1 (m7), CS-35[12]-2 (m12), R1[7]-5 (p7), and the unselected Ph.D.-7 library (US), as indicated at the bottom of the corresponding bars. The results are expressed as the means \pm SDs for three independent experiments. Asterisks represent significance levels (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$) relative to the results for the unselected control for each set of sera.

dependent approaches were used, at least one sequence was selected by both procedures. Among the various groups displayed in Table 2, the second group consisted of a sequence that recurred most frequently. In this group, the sequences of six of the nine phage clones (R1[7]-5 to R1[7]-10) were identical. The remaining three clones (clones R1[7]-11, R1[7]-12, and R1[7]-13) differed in the last three amino acids. It is apparent from the comparison that the sequences present in this group represented a strong consensus.

Selected phage clones preferentially bind to anti-BCG rabbit immune sera. Both of the panning experiments resulted in a large collection of phage clones that represent mimotopes of mycobacterial surface antigens. Two of these appeared to mimic LAM, as they were obtained by panning on a defined MAb against LAM, MAb CS-35. The rest were derived without prior knowledge of the antigens. The common feature expected to be shared by all these phagotopes is preferential reactivity toward *M. bovis* BCG-immunized rabbit sera compared to that to preimmune sera. Three phage clones were selected for initial testing: CS-35[7]-1 (which displayed the sequence picked up both by panning on MAb CS-35 and by panning on R1 polyclonal serum), CS-35[12]-2, and R1[7]-5 (which represented the most frequently recurring sequence in the second group shown in Table 2). Reverse phage ELISA was performed with the three preimmune and immune rabbit serum samples. The unselected phage library control should not have any difference in reactivity with preimmune and immune sera. Whatever difference may surface must be considered the background. The differential reactivity in the case of

the selected phage clones should be higher than that of the unselected phage library.

The results (Fig. 4) show that all the three clones gave significantly higher ($P \leq 0.05$) differential reactivities (difference in the absorbance [OD_{405}] of the immune sera from that of the preimmune sera [ΔOD_{405}]) compared to that of the unselected Ph.D.-7 library for all the three rabbit sera. However, it may be noted that in the case of the R1-selected phagotopes, CS-35[7]-1 and R1[7]-5, there was a higher level of binding to R1 sera.

The panning of the Ph.D.-7 library on anti-BCG R1 serum yielded not only the 7-mer LAM mimotope displaying phage clone R1[7]-1 (which is the same as CS-35[7]-1 obtained from panning on MAb CS-35) and R1[7]-5 (which represented the most frequently recurring sequence in the second group shown in Table 2) but also several other groups of clones displaying different enriched sequences (Table 2). Phage clones R1[7]-11, -13, -14, -17, -19, and -21 to -24, representing various other groups from Table 2, were subjected to reverse phage ELISA. Phage R1[7]-5 was used as the positive control, as it reacted positively with all the three rabbit immune sera (as shown in Fig. 4), and the unselected Ph.D.-7 library was used as the negative control. Since the R1 serum was used for panning purposes, to prevent any bias, this serum was not used in this experiment and only R2 (Fig. 5A) and R3 (Fig. 5B) sera were used. Each clone was reacted with the immune and the corresponding preimmune sera, and the differences in reactivity (ΔOD_{405}) were determined. The results (Fig. 5) show that by and large all the clones were reactive to both sera, except

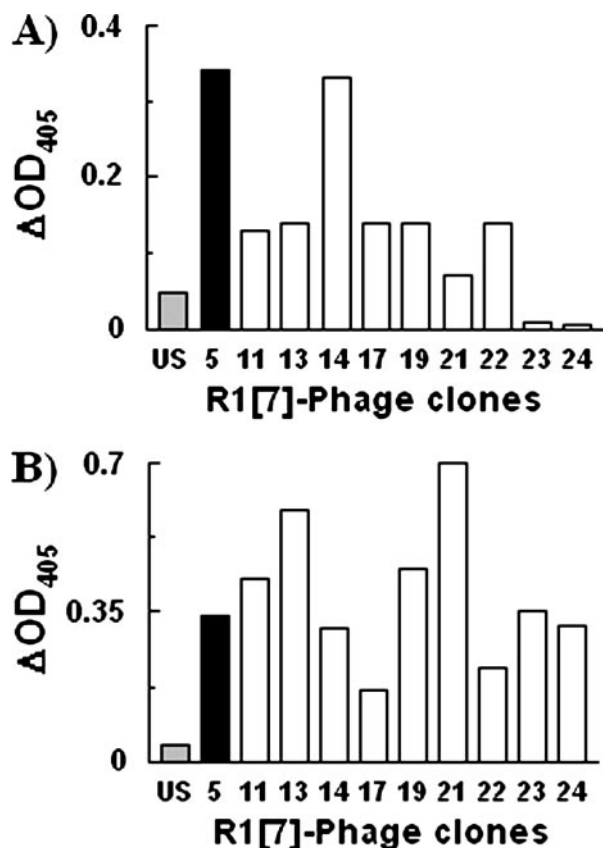


FIG. 5. Reactivities of the other enriched phage clones obtained by panning on R1 serum to R2 and R3 sera. Reverse phage ELISA was done with R2 (A) and R3 (B) preimmune and immune sera (1:40 dilution). Phage R1[7]-5 was used as the positive control (black bars), and the unselected Ph.D.-7 library (US) was used as the negative control (gray bars). The bars represent the ΔOD at 405 nm for the immune sera and the preimmune sera in the case of the selected R1[7] clones, as indicated at the bottom of the corresponding bars) and the unselected library.

R1[7]-21, -23, and -24, which seemed to have a preference for the R3 rabbit serum.

Binding affinity of the synthetic peptide corresponding to the 7-mer LAM mimotope for MAb CS-35. The peptide corresponding to the 7-mer LAM mimotope that was identified, referred to as PepCS-35[7], was synthesized chemically. The K_D for PepCS-35[7] binding to purified MAb CS-35 was determined by the tryptophanyl fluorescence enhancement method. As PepCS-35[7] has no tryptophan residue, it could be used as an enhancer, but a similar exercise could not be done in the case of PepCS-35[12], the synthetic peptide representing the 12-mer mimotope, as tryptophan forms a part of the sequence. Figure 6 shows the dose-dependent tryptophan fluorescence enhancement profile of MAb CS-35 (0.3 μM) upon its binding to PepCS-35[7]. The data were fitted to the single-site binding equation (equation 1), and K_D was determined. The K_D value thus obtained was found to be $7.55 \pm 0.83 \mu M$ (SEM) ($P = 1.09 \times 10^{-7}$).

Dot blot analysis of LAM peptide mimotopes for detection of anti-LAM antibody in TB patient sera. Since an expected application of these LAM mimotopes will be to replace LAM

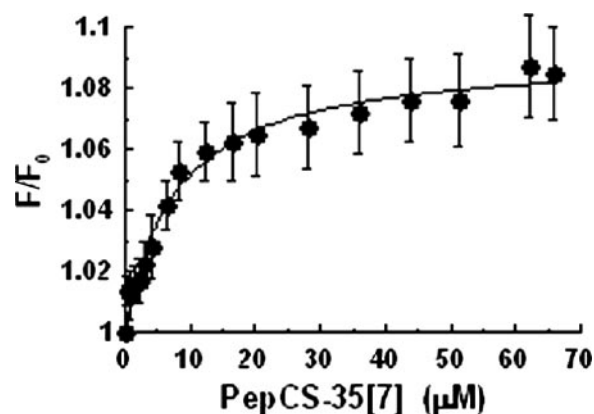


FIG. 6. Determination of the dissociation constant of PepCS-35[7] binding to MAb CS-35. Binding of PepCS-35[7] to purified MAb CS-35 was determined from the dose-dependent enhancement in the intrinsic tryptophanyl fluorescence of MAb CS-35 (0.3 μM) in PBS at room temperature. F/F_0 values are means \pm SEMs (indicated by the error bars) of three independent experiments. The excitation and emission were 295 and 333 nm, respectively. In the plot, F_0 is the initial fluorescence and F is the fluorescence obtained at a particular concentration of peptide.

for the diagnosis of TB, it was considered important to make a preliminary evaluation of their ability to detect anti-LAM antibody in TB patient sera. To develop a convenient peptide-based assay as a potential probe to screen sera from TB patients for the presence of LAM-specific antibodies, dot blotting was attempted with these peptides, with LAM used as the positive control. To test the feasibility of using dot blotting with LAM peptide mimotopes, the three rabbit anti-BCG sera were initially screened. The results showed that LAM as well as both the peptides, PepCS-35[7] (Fig. 7B) and PepCS-35[12] (Fig. 7A), cross-reacted with the three rabbit immune sera but not with the corresponding preimmune sera.

After the dot blot setup was established with rabbit sera, we attempted a similar experiment with human sera. For this, we selected five TB patient serum samples which showed distinguishably higher (approximately fourfold) signals in the LAM ELISA than the five healthy control serum samples. Both LAM and the two mimotopic peptides were used, as before, and the results (Fig. 7C) showed that for the TB patient sera, the spots were visibly more intense than those for the healthy control sera. The bar diagram corresponding to the densitometric scan of this blot, represented in Fig. 7D, shows that the average cross-reactivity of LAM with TB patient sera was approximately 16-fold higher than that with the healthy control sera, whereas the average cross-reactivity of PepCS-35[12] and PepCS-35[7] with the TB patient sera was approximately 8-fold higher than that with the healthy control sera. This showed that the peptides identified mimic LAM in their ability to bind to anti-LAM antibody in sera from TB patients, indicating the possibility that the identified mimotopes could have an application in LAM-based diagnostic assays.

Immunomodulatory role of LAM mimotopes. Lipoarabinomannan, a major cell wall component of mycobacteria, is known to be a strong immunomodulator (2); in this capacity, it plays a key role in the pathogenesis of TB (53). LAM can be capped with either mannose (ManLAM) or phosphoinositol

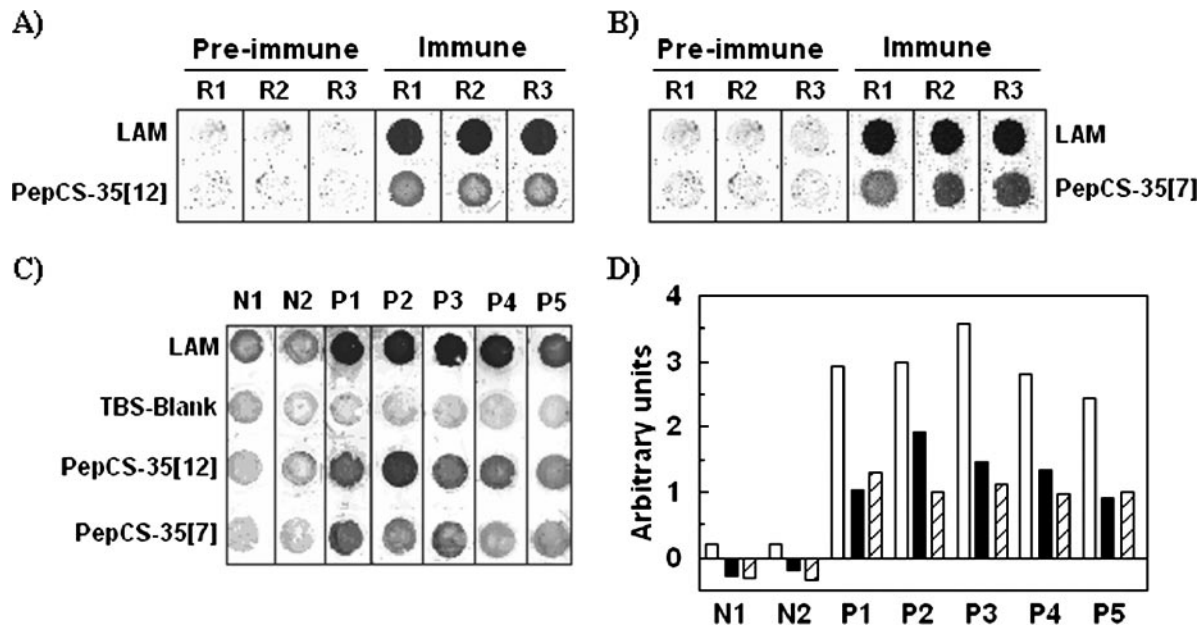


FIG. 7. Dot blot analysis of LAM peptide mimotopes for detection of anti-LAM antibody. *M. tuberculosis* H37Rv LAM (0.1 µg per well), PepCS-35[7] (5 µg per well) (B), or PepCS-35[12] (5 µg per well) (A) was immobilized onto an Immobilon-PS^Q PVDF membrane, and immunoblotting was done with the three rabbit sera (R1, R2 and R3; preimmune or immune) (A and B) and human sera (C). The rabbit sera were used at 1:500 dilutions, and the human sera were used at 1:100 dilutions. The TB patient sera are designated P1 to P5, and the healthy control sera are designated N1 and N2. LAM was used as a positive control, and TBS buffer was used as a negative control. The blots shown in panel C were scanned densitometrically, and the spot intensities of LAM (white bars), PepCS-35[12] (black bars), and PepCS-35[7] (striped bars), after blank subtraction, were represented as the bar diagram in panel D. The data presented are representative of one of three independent dot blots, with similar results obtained in each dot blot.

(PILAM). The former is particularly known to be predominant in virulent strains of *M. tuberculosis* (10), whereas LAM from the fast-growing saprophyte *M. smegmatis* is capped with phosphoinositol. PILAM is more efficient at releasing proinflammatory cytokines (41, 60), particularly TNF- α (9), than ManLAM. As the peptides appear to be the mimics of LAM, as evident from their cross-reactivity with MAb CS-35, it is possible that these may have immunomodulatory activity.

To investigate this possibility, the effect of adding PILAM and ManLAM (at a fixed concentration of 5 µg/ml) on the cytokine release profile from a murine macrophage cell line, J774A.1, as well as from primary mouse BMM ϕ was monitored. The choice of this concentration was based on the findings of a previous report (9), in which LAMs were effectively used at a concentration range of 1 to 10 µg/ml. The increase in cytokine content was represented as the Δ OD at 492 nm for the supernatant from the stimulated cells and the control cells (unstimulated). Consistent with previous reports (41) PILAM was found to be more active than ManLAM in releasing TNF- α and IL-12 from both J774A.1 and primary mouse BMM ϕ (Fig. 8A and B).

Having tested the systems with the two types of LAMs, the peptides were then used to assess their abilities to release the same cytokines. The results show that the release of both cytokines, TNF- α (Fig. 8C and E) and IL-12 (Fig. 8D and F), was stimulated by both peptides, PepCS-35[7] and PepCS-35[12], in a dose-dependent manner (up to 10 µM) in J774A.1 macrophages (Fig. 8C and D) as well as BMM ϕ (Fig. 8E and F). The activity of PepCS-35[12] appears to be marginally greater than that of PepCS-35[7].

DISCUSSION

The present investigation explores the possibility of applying the phage display technique in the context of deriving peptide mimotopes of mycobacterial antigens. The working hypothesis is that the peptide mimics of mycobacterial antigens, particularly the nonprotein ones, would be useful as diagnostic agents. Moreover, since mycobacterial antigens are known to be immunomodulators (7, 8), in addition to serving as diagnostic tools, such peptide mimics could be useful for therapeutic purposes, such as for the treatment of cancer (42).

This is one of the first reports in which phage display tools have been used to identify mimotopes of mycobacterial antigens. An investigation on a somewhat similar theme has been attempted earlier (18). In that investigation, antiserum against extracted mycobacterial cell surface sugar was used to obtain peptide mimotopes. The search resulted in certain sequences which appeared to be mimics of LAM; however, the mimicry was not confined to LAM but extended to other non-LAM oligomannosylated structures. The use of extracted sugars to raise antibodies may be disadvantageous, since the extracted molecules are robbed of their surroundings. Given that the structure of an antigen is likely to be influenced by its surroundings, or "landscape" (37), the use of antibodies against whole cells should be a better option. In this study we have used two approaches. One of these was a targeted approach in which an epitope-specific MAb against LAM was used, and the other was a random approach in which antiserum raised against whole cells was used for panning. The rationale behind the second approach was that such an antiserum would contain

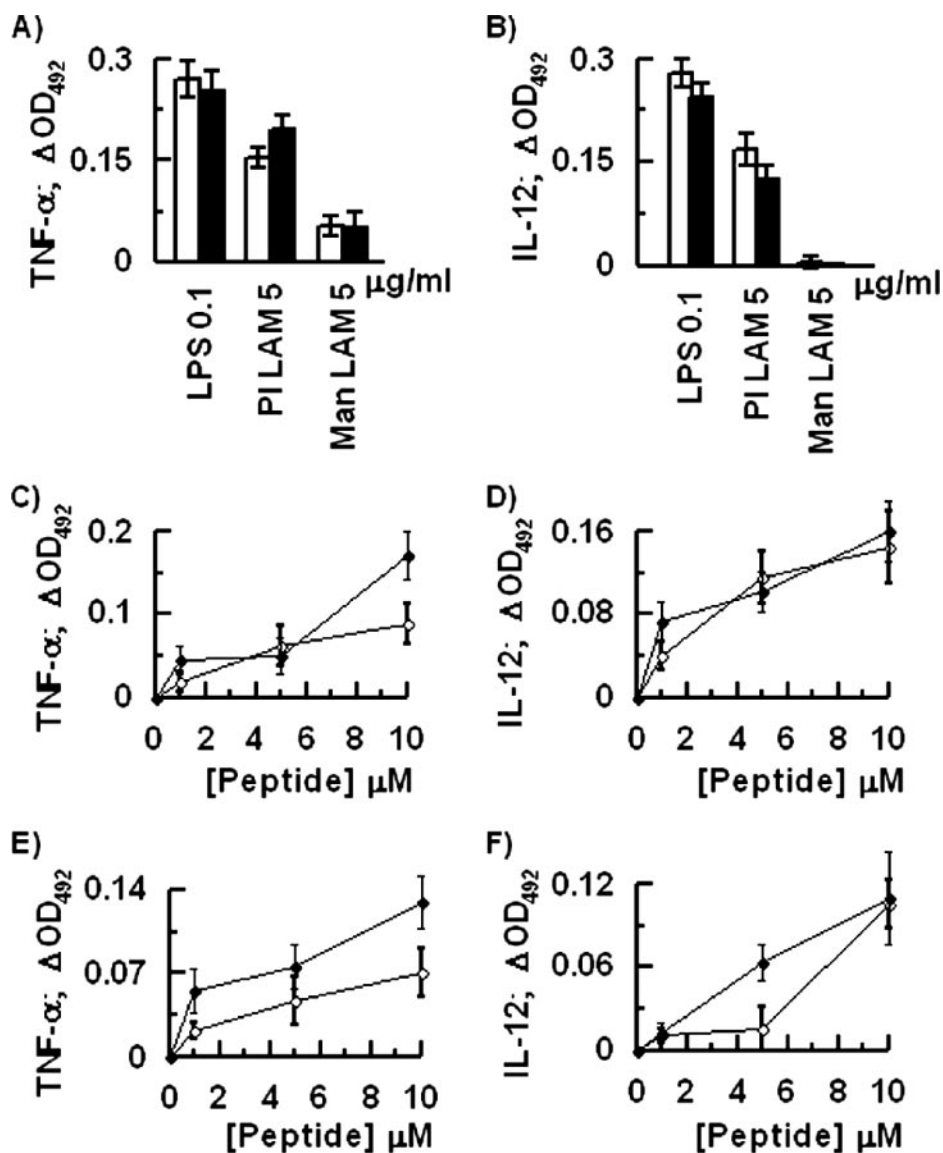


FIG. 8. Stimulatory effects of peptides on cytokine release. J774A.1 cells or BMMφ (2×10^5 to 3×10^5 cells/well; two replicates per treatment) were incubated with medium alone, medium plus LPS (0.1 μg/ml), medium plus PILAM (5 μg/ml), medium plus ManLAM (5 μg/ml), or medium plus increasing concentrations (1, 5, and 10 μM) of Pep CS-35[7] or Pep CS-35[12], as indicated. Culture supernatants were collected and examined for cytokine release by sandwich ELISA after 24 h, as described in Materials and Methods. (A and B) TNF-α and IL-12 release, respectively, from either J774A.1 cells (white bars) or BMMφ (black bars) in response to LPS, PILAM, and ManLAM, as indicated. (C to F) Dose-dependent cytokine release (TNF-α and IL-12) from J774A.1 cells (C and D) and BMMφ (E and F), after stimulation with the peptides PepCS-35[7] (white circles) and PepCS-35[12] (black circles). The data are represented as the ΔOD at 492 nm of the supernatant from the stimulated cells and that from the control cells (cells incubated with medium only without the addition of any stimulant). The results are expressed as the means ± SDs for three independent experiments.

a high level of representation of antibodies against surface antigens presented in their natural context.

Panning on the MAb yielded two LAM-specific mimotopes, one 7-mer and the other 12-mer, whereas panning on the anti-BCG polyclonal serum yielded a large collection of 7-mer mimotopes that reacted specifically with independently derived anti-BCG immune sera. Several groups of peptide sequences could be identified. Examination of the nucleotide sequences encoding the peptides revealed a bias in the codon usage. It was observed that stretches comprising three or more invariant amino acids were identically coded. This may be partly due to

the fact that 32 instead of 64 possible codons were used in the construction of the libraries and partly, perhaps more plausibly, due to codon pair biases (19), which may have been introduced during successive amplification steps.

The groups of peptide sequences obtained may be expanded further if more phage clones are sequenced. The phage pool derived after the last round of panning on the polyclonal serum therefore represents a vast source of peptide mimotopes of mycobacterial antigens. The frequency with which each sequence was picked up is likely to be directly proportional to its affinity for the antiserum, although this has not been specifi-

cally tested. One of the mimotopic sequences identified by panning on the polyclonal serum was found to be identical to the 7-mer LAM mimotope raised on MAb CS-35. The observed convergence between the two approaches is an indirect validation of the methods used for the mining of mycobacterial mimotopes. Phagotopes CS-35[7]-1 and CS-35[12]-2 reacted specifically with MAb CS-35. MAb CS-35 is known to cross-react with the hexaarabinofuranosyl motif of LAM from *M. tuberculosis* and other mycobacteria (28), and hence, the 7-mer and the 12-mer MAb CS-35-derived peptides appear to mimic the hexaarabinofuranosyl residues rather than the mannosyl residues, as was the case with the mimotopes reported in an earlier work in this area (18). A substantially accurate K_D for the interaction of the peptide representing the 7-mer mimotope with MAb CS-35 was obtained. When the K_D value of 7.55 μM was converted to the association constant (K_a), a value of $\sim 1 \times 10^5 \text{ M}^{-1}$ was obtained. This value is comparable to those of the binding constants for several antigen-antibody interactions (39, 56). A K_D value for the peptide corresponding to the 12-mer mimotope could not be derived by the fluorescence-based method due to the problem of intrinsic tryptophanyl fluorescence. The results of reverse phage ELISA experiments, however, suggest that CS-35[12]-2 reacted more strongly with the MAb than CS-35[7]-1.

Both CS-35[7]-1 and CS-35[12]-2 cross-reacted with all three rabbit anti-BCG immune sera, but CS-35[7]-1 had a bias toward the R1 serum. It appears that in the case of screening on polyclonal antibody, the selected mimotopes are likely to have a disproportionately higher affinity toward the sera from which they were derived, probably due to a better fit into the antigen binding cavity. Such a bias, if any, can be eliminated by testing their ability to interact with the independently derived antisera, as was done in this study.

The immunodominant properties of LAM and other cell surface mycobacterial antigens lead to the stimulation of antibody production in infected animals and humans, because of which such antigens have been incorporated into ELISA for the detection of antibodies, which serve as an indication of mycobacterial infection (5, 13). LAM has been used in various diagnostic kits (40), and therefore, it should be possible to replace LAM with the peptides. This will make it easier to manufacture diagnostic kits. We have attempted to develop a convenient dot blot assay with the LAM mimotopes identified to screen sera from TB patients for the presence of LAM-specific antibodies. We deliberately chose a panel of TB patient sera with high anti-LAM antibody titers and tested whether the peptides gave positive signals with these sera in a dot blot assay. The results show an acceptable correlation between reactivity against LAM and the signals for the peptides. This indicates that these peptides, and also possibly several others derived from the mimotopic library reported here, could be used in various diagnostic formats ranging from conventional ELISAs to the more complex combinatorial "chip"-based systems (34, 52). The use of combinatorial systems with mimotopes could also help in overcoming the variability factor linked with the TB ELISA (32, 40).

The potential application of these peptides goes beyond diagnostics. Immunomodulation is an important option in the prevention of many diseases, such as cancer (57). The use of mycobacteria for the treatment of tuberculosis and certain

forms of cancer is well known (42, 51, 55). Although the precise mechanisms of action are probably not known, it is most likely that modulation of cytokine responses is a key to such immunotherapy. Since the peptide mimotopes obtained in this study appear to mimic immunoactive mycobacterial antigens, their ability to stimulate cytokine release from macrophages was assessed. We have currently focused on the LAM mimotopes, as immunomodulation by LAM is a fairly well studied phenomenon (2). Both the LAM mimotopic peptides were found to stimulate the release of TNF- α and IL-12 from J774A.1 cells as well as BMM ϕ . TNF- α is an essential cytokine required for protection against *M. tuberculosis* and other pathogens. It increases the intracellular killing of pathogens by macrophages and also stimulates granuloma formation (9). IL-12 is known to play a major role in potent T-cell-dependent protection against infection with intracellular pathogens, including *Mycobacterium* spp. (60). The stimulatory effect of PepCS-35[12] appears to be marginally more than that of PepCS-35[7], particularly in the context of TNF- α . The significance of this is not clear as yet but may be due to different affinities of the peptides for the LAM receptors on the macrophages. Interestingly, reverse phage ELISA also showed the higher reactivity of PepCS-35[12] with CS-35; thus, there may be a correlation between the affinities for MAb and the cytokine-stimulatory capacities of the peptides. The mechanism of action of the peptides has not been established yet, but most likely, they function by mimicking the hexaarabinofuranosyl motif of LAM.

We have also tested whether the peptide mimotopes stimulate T cells. A gamma interferon-based assay was performed (data not shown) with peripheral blood mononuclear cells obtained from limited cohorts of TB patients and healthy individuals. Although the peripheral blood mononuclear cells could be stimulated by concanavalin A mitogen and crude extracts of mycobacteria, neither the LAM nor the peptides gave any significant stimulation. The LAM mimotopic peptides, unlike antigens such as ESAT-6 (24), do not appear to evoke T-cell responses. It is, however, possible that the other peptides in the panel could be potential stimulators of mycobacterium-reactive T cells.

In conclusion, the peptide mimotopes derived in the present study may have a wide range of applications, such as the following: (i) they could replace corresponding antigens in diagnostic assays, leading to the development of novel combinatorial diagnostic chips; (ii) they may be used as immunomodulatory agents in specific contexts, such as cancer therapy; and (iii) because they are proinflammatory, they could be used as adjuvants in vaccines. Given the complexity of tuberculosis, it is difficult to obtain a solution in a single study; however, the information provided here could give valuable leads to future applications of the phage display to TB. It would be interesting to apply the phage display technique directly to TB patients. By using suitable healthy controls and TB patients, it should be possible to perform a subtractive search for new mimotopes related to *M. tuberculosis*.

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REFERENCES

- Andersen, P. 2001. TB vaccines: progress and problems. *Trends Immunol.* **22**:160–168.
- Barnes, P. F., D. Chatterjee, J. S. Abrams, S. Lu, E. Wang, M. Yamamura, P. J. Brennan, and R. L. Modlin. 1992. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan. Relationship to chemical structure. *J. Immunol.* **149**:541–547.
- Barry, C. E., III. 2001. Interpreting cell wall 'virulence factors' of *Mycobacterium tuberculosis*. *Trends Microbiol.* **9**:237–241.
- Besra, G. S., and D. Chatterjee. 1994. Lipids and carbohydrates of *Mycobacterium tuberculosis*, p. 285–306. In B. R. Bloom (ed.), *Tuberculosis: pathogenesis, protection, and control*. American Society for Microbiology, Washington, D.C.
- Bothamley, G. H. 1995. Serological diagnosis of tuberculosis. *Eur. Respir. J. Suppl.* **20**:676s–688s.
- Brandt, L., M. Elhay, I. Rosenkrands, E. B. Lindblad, and P. Andersen. 2000. ESAT-6 subunit vaccination against *Mycobacterium tuberculosis*. *Infect. Immun.* **68**:791–795.
- Brennan, P. J., and H. Nikaido. 1995. The envelope of mycobacteria. *Annu. Rev. Biochem.* **64**:29–63.
- Chatterjee, D. 1997. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. *Curr. Opin. Chem. Biol.* **1**:579–588.
- Chatterjee, D., A. D. Roberts, K. Lowell, P. J. Brennan, and I. M. Orme. 1992. Structural basis of capacity of lipoarabinomannan to induce secretion of tumor necrosis factor. *Infect. Immun.* **60**:1249–1253.
- Chatterjee, D., K. Lowell, B. Rivoire, M. R. McNeil, and P. J. Brennan. 1992. Lipoarabinomannan of *Mycobacterium tuberculosis*. Capping with mannose residues in some strains. *J. Biol. Chem.* **267**:6234–6239.
- Chiang, I. H., J. Suo, K. J. Bai, T. P. Lin, K. T. Luh, C. J. Yu, and P. C. Yang. 1997. Serodiagnosis of tuberculosis. A study comparing three specific mycobacterial antigens. *Am. J. Respir. Crit. Care Med.* **156**:906–911.
- Cunto-Amesty, G., P. Luo, B. Monzavi-Karbassi, A. Lees, J. Alexander, M. F. del Guercio, M. H. Nahm, C. Artaud, J. Stanley, and T. Kieber-Emmons. 2003. Peptide mimotopes as prototype templates of broad-spectrum surrogates of carbohydrate antigens. *Cell. Mol. Biol.* **49**:245–254.
- Daniel, T. M., and S. M. Debanne. 1987. The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay. *Am. Rev. Respir. Dis.* **135**:1137–1151.
- Denis, O., A. Tanghe, K. Palfiet, F. Jurion, T. P. van den Berg, A. Vanonckelen, J. Ooms, E. Saman, J. B. Ulmer, J. Content, and K. Huygen. 1998. Vaccination with plasmid DNA encoding mycobacterial antigen 85A stimulates a CD4⁺ and CD8⁺ T-cell epitopic repertoire broader than that stimulated by *Mycobacterium tuberculosis* H37Rv infection. *Infect. Immun.* **66**:1527–1533.
- Dybwad, A., O. Forre, J. Kjeldsen-Kragh, J. B. Natvig, and M. Sioud. 1993. Identification of new B cell epitopes in the sera of rheumatoid arthritis patients using a random nanopeptide phage library. *Eur. J. Immunol.* **23**:3189–3193.
- Ehlers, M. R., and M. Daffe. 1998. Interactions between *Mycobacterium tuberculosis* and host cells: are mycobacterial sugars the key? *Trends Microbiol.* **6**:328–335.
- Ewer, K., P. Cockle, S. Gordon, H. Mansoor, M. Govaerts, K. Walravens, S. Marche, G. Hewinson, and M. Vordermeier. 2006. Antigen mining with iterative genome screens identifies novel diagnostics for the *Mycobacterium tuberculosis* complex. *Clin. Vaccine Immunol.* **13**:90–97.
- Gevorkian, G., E. Segura, G. Acero, J. P. Palma, C. Espitia, K. Manoutcharian, and L. M. Lopez-Marin. 2005. Peptide mimotopes of *Mycobacterium tuberculosis* carbohydrate immunodeterminants. *Biochem. J.* **387**:411–417.
- Gutman, G. A., and G. W. Hatfield. 1989. Nonrandom utilization of codon pairs in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **86**:3699–3703.
- Hammasur, B., G. Kallenius, and S. B. Svenson. 1999. A new rapid and simple method for large-scale purification of mycobacterial lipoarabinomannan. *FEMS Immunol. Med. Microbiol.* **24**:11–17.
- Hammasur, B., J. Bruchfeld, M. Haile, A. Pawlowski, B. Bjorvatn, G. Kallenius, and S. B. Svenson. 2001. Rapid diagnosis of tuberculosis by detection of mycobacterial lipoarabinomannan in urine. *J. Microbiol. Methods* **45**:41–52.
- Hetland, G., H. G. Wiker, K. Hogasen, B. Hammasur, S. B. Svenson, and M. Harboe. 1998. Involvement of antilipoarabinomannan antibodies in classical complement activation in tuberculosis. *Clin. Diagn. Lab. Immunol.* **5**:211–218.
- Horwitz, M. A., B. W. Lee, B. J. Dillon, and G. Harth. 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. USA* **92**:1530–1534.
- Johnson, P. D., R. L. Stuart, M. L. Grayson, D. Olden, A. Clancy, P. Ravn, P. Andersen, W. J. Britton, and J. S. Rothel. 1999. Tuberculin-purified protein derivative-, MPT-64-, and ESAT-6-stimulated gamma interferon responses in medical students before and after *Mycobacterium bovis* BCG vaccination and in patients with tuberculosis. *Clin. Diagn. Lab. Immunol.* **6**:934–937.
- Jolley, M. E., S. Rudikoff, M. Potter, and C. P. Glaudemans. 1973. Spectral changes on binding of oligosaccharides to murine immunoglobulin A myeloma proteins. *Biochemistry* **12**:3039–3044.
- Julian, E., L. Matas, A. Perez, J. Alcaide, M. A. Lanelle, and M. Luquin. 2002. Serodiagnosis of tuberculosis: comparison of immunoglobulin A (IgA) response to sulfolipid I with IgG and IgM responses to 2,3-diacetylglucose, 2,3,6-triacetylglucose, and cord factor antigens. *J. Clin. Microbiol.* **40**:3782–3788.
- Julian, E., L. Matas, J. Alcaide, and M. Luquin. 2004. Comparison of antibody responses to a potential combination of specific glycolipids and proteins for test sensitivity improvement in tuberculosis serodiagnosis. *Clin. Diagn. Lab. Immunol.* **11**:70–76.
- Kaur, D., T. L. Lowary, V. D. Vissa, D. C. Crick, and P. J. Brennan. 2002. Characterization of the epitope of anti-lipoarabinomannan antibodies as the terminal hexaarabinofuranosyl motif of mycobacterial arabinans. *Microbiol. J.* **148**:3049–3057.
- Kitada, S., R. Maekura, N. Toyoshima, N. Fujiwara, I. Yano, T. Ogura, M. Ito, and K. Kobayashi. 2002. Serodiagnosis of pulmonary disease due to *Mycobacterium avium* complex with an enzyme immunoassay that uses a mixture of glycopeptidolipid antigens. *Clin. Infect. Dis.* **35**:1328–1335.
- Kouzmitcheva, G. A., V. A. Petrenko, and G. P. Smith. 2001. Identifying diagnostic peptides for Lyme disease through epitope discovery. *Clin. Diagn. Lab. Immunol.* **8**:150–160.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
- Lyashchenko, K., R. Colangeli, M. Houde, H. Al Jahdali, D. Menzies, and M. L. Gennaro. 1998. Heterogeneous antibody responses in tuberculosis. *Infect. Immun.* **66**:3936–3940.
- Mintz, P. J., J. Kim, K. A. Do, X. Wang, R. G. Zinner, M. Cristofanilli, M. A. Arap, W. K. Hong, P. Troncoso, C. J. Logothetis, R. Pasqualini, and W. Arap. 2003. Fingerprinting the circulating repertoire of antibodies from cancer patients. *Nat. Biotechnol.* **21**:57–63.
- Noya, O., and B. Alarcon de Noya. 1998. The multiple antigen blot assay (MABA): a simple immunoenzymatic technique for simultaneous screening of multiple antigens. *Immunol. Lett.* **63**:53–56.
- Onyebujoh, P., A. Zumla, I. Ribeiro, R. Rustomjee, P. Mwaba, M. Gomes, and J. M. Grange. 2005. Treatment of tuberculosis: present status and future prospects. *Bull. W. H. O.* **83**:857–865.
- Parra, M., N. Cadieux, T. Pickett, V. Dheenadhayalan, and M. J. Brennan. 2006. A PE protein expressed by *Mycobacterium avium* is an effective T-cell immunogen. *Infect. Immun.* **74**:786–789.
- Petrenko, V. A., G. P. Smith, X. Gong, and T. Quinn. 1996. A library of organic landscapes on filamentous phage. *Protein Eng.* **9**:797–801.
- Pieters, J. 2001. Entry and survival of pathogenic mycobacteria in macrophages. *Microbes Infect.* **3**:249–255.
- Pollet, R., and H. Edelhoch. 1973. The binding properties of anti-phosphorylcholine mouse myeloma proteins as measured by protein fluorescence. *J. Biol. Chem.* **248**:5443–5447.
- Pottumarthy, S., V. C. Wells, and A. J. Morris. 2000. A comparison of seven tests for serological diagnosis of tuberculosis. *J. Clin. Microbiol.* **38**:2227–2231.
- Quesniaux, V. J., D. M. Nicolle, D. Torres, L. Kremer, Y. Guerardel, J. Nigou, G. Puzo, F. Erard, and B. Riffe. 2004. Toll-like receptor 2 (TLR2)-dependent-positive and TLR2-independent-negative regulation of proinflammatory cytokines by mycobacterial lipomannans. *J. Immunol.* **172**:4425–4434.
- Raez, L. E., S. Fein, and E. R. Podack. 2005. Lung cancer immunotherapy. *Clin. Med. Res.* **3**:221–228.
- Raja, A., K. R. Uma Devi, B. Ramalingam, and P. J. Brennan. 2002. Immunoglobulin G, A, and M responses in serum and circulating immune complexes elicited by the 16-kilodalton antigen of *Mycobacterium tuberculosis*. *Clin. Diagn. Lab. Immunol.* **9**:308–312.
- Rosseels, V., S. Marche, V. Roupie, M. Govaerts, J. Godfroid, K. Walravens, and K. Huygen. 2006. Members of the 30- to 32-kilodalton mycolyl transferase family (Ag85) from culture filtrate of *Mycobacterium avium* subsp. *paratuberculosis* are immunodominant Th1-type antigens recognized early upon infection in mice and cattle. *Infect. Immun.* **74**:202–212.
- Saha, A., A. Sharma, A. Dhar, B. Bhattacharyya, S. Roy, and S. K. Das Gupta. 2005. Antagonists of Hsp16.3, a low-molecular-weight mycobacterial chaperone and virulence factor, derived from phage-displayed peptide libraries. *Appl. Environ. Microbiol.* **71**:7334–7344.
- Scalati, G., X. Chen, W. Liu, J. N. Telles, O. J. Cohen, M. Vaccarezza, T. Igarashi, and A. S. Fauci. 1999. Selection of HIV-specific immunogenic epitopes by screening random peptide libraries with HIV-1-positive sera. *J. Immunol.* **162**:6155–6161.
- Scarpellini, P., S. Tasca, L. Galli, A. Beretta, A. Lazzarin, and C. Fortis.

2004. Selected pool of peptides from ESAT-6 and CFP-10 proteins for detection of *Mycobacterium tuberculosis* infection. *J. Clin. Microbiol.* **42**:3469–3474.
48. Schluger, N. W., and W. N. Rom. 1998. The host immune response to tuberculosis. *Am. J. Respir. Crit. Care Med.* **157**:679–691.
49. Skjot, R. L., T. Oettinger, I. Rosenkrands, P. Ravn, I. Brock, S. Jacobsen, and P. Andersen. 2000. Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens. *Infect. Immun.* **68**:214–220.
50. Smith, G. P., and V. A. Petrenko. 1997. Phage display. *Chem. Rev.* **97**:391–410.
51. Stanford, J., C. Stanford, and J. Grange. 2004. Immunotherapy with *Mycobacterium vaccae* in the treatment of tuberculosis. *Front. Biosci.* **9**:1701–1719.
52. Steinhauer, C., C. Wingren, A. C. Hager, and C. A. Borrebaeck. 2002. Single framework recombinant antibody fragments designed for protein chip applications. *BioTechniques Suppl.* 2002: 38–45.
53. Strohmeier, G. R., and M. J. Fenton. 1999. Roles of lipoarabinomannan in the pathogenesis of tuberculosis. *Microbes Infect.* **1**:709–717.
54. Tessema, T. A., G. Bjune, B. Hamasur, S. Svenson, H. Syre, and B. Bjorvatn. 2002. Circulating antibodies to lipoarabinomannan in relation to sputum microscopy, clinical features and urinary anti-lipoarabinomannan detection in pulmonary tuberculosis. *Scand. J. Infect. Dis.* **34**:97–103.
55. Tishler, M., and Y. Shoenfeld. 2006. BCG immunotherapy—from pathophysiology to clinical practice. *Expert Opin. Drug Safety* **5**:225–229.
56. Vyas, N. K., M. N. Vyas, M. C. Chervenak, D. R. Bundle, B. M. Pinto, and F. A. Quijcho. 2003. Structural basis of peptide-carbohydrate mimicry in an antibody-combining site. *Proc. Natl. Acad. Sci. USA* **100**:15023–15028.
57. Waldmann, T. A. 2006. Effective cancer therapy through immunomodulation. *Annu. Rev. Med.* **57**:65–81.
58. Wilkinson, R. J., K. Haslov, R. Rappuoli, F. Giovannoni, P. R. Narayanan, C. R. Desai, H. M. Vordermeier, J. Paulsen, G. Pasvol, J. Ivanyi, and M. Singh. 1997. Evaluation of the recombinant 38-kilodalton antigen of *Mycobacterium tuberculosis* as a potential immunodiagnostic reagent. *J. Clin. Microbiol.* **35**:553–557.
59. World Health Organization. 2005. Tuberculosis. Fact sheet no. 104. World Health Organization, Geneva, Switzerland. [Online.] <http://www.who.org>.
60. Yoshida, A., and Y. Koide. 1997. Arabinofuranosyl-terminated and mannosylated lipoarabinomannans from *Mycobacterium tuberculosis* induce different levels of interleukin-12 expression in murine macrophages. *Infect. Immun.* **65**:1953–1955.