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 μM. 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.

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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 Nterminal region of the pII1 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 pII1 sequence. The Ph.D.-7 library consisted of 2.8 × 10^10 independent clones, sufficient to encode most, if not all, of the 20, or 1.25 × 10^9, possible 7-residue sequences; and the Ph.D.-12 library consisted of 1.9 × 10^10 independent clones, sufficient to encode 20^12, or 4.1 × 10^13, 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 Dickenson and Co. [BD]) supplemented with 10% oleate-albumin-dextrose-catalase enrichment (Difco, BD, USA), 0.5% glycerol, and 0.2% Tween 80 until 80% of the cells were killed at 37°C with continuous shaking. Mycobacterium smegmatis LR222 was grown at 37°C under shaking conditions in 2 liters of phosphate-buffered saline (PBS; 15 mM sodium phosphate buffer [pH 9.1]). The bacterial suspension was diluted for two subcutaneous (s.c.) injections (2 × 10^7 cells/ml) and four intravenous (i.v.) injections (dosing increases 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 prebleeding was done immediately prior to immunization with subcutaneous injections of the fourth library. Serum was 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 were 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/ml) 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-P 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. 1987). The membranes were developed with 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) ready-to-use substrate solution (providing 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 identify the antibody-phyge 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 NaHCO_3 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 10^6 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 and purified by double precipitation in the cold with 1/10 volume of polyethylene glycol (PEG)-NaCl (20% [vol/vol] polyethylene glycol 8000, 2.5 M NaCl), and used for a second round of biopanning. The phage concentration was increased by 10fold each time. The biopanning was performed by raising the Tewn 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

**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 N01-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 Hamaur 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 amebocyte assay.
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-RBC rabbit polyclonal serum, except that 1 μl 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 μl 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-choro-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 Genie 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-BCC polyclonal sera. Briefly, either 200 μl/well of the MAb tissue culture supernatant, a 1:40 dilution of the rabbit serum, or 1 μl/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 Genie Pvt. Ltd., Peenya, Bangalore, India). The unrelated MAb CS-49, which is reactive against HspX (Rv3013c) 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-prepitation method, were added to each coated well (1010 to 1011 PFU/well), and the plates were incubated for 2 h at room temperature. Unbound phage was removed by washing with TBS (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-ethylthiazoline-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 μg/well) diluted in 0.05 M Na2CO3-NaHCO3 buffer (pH 9.6) and blocked with 5% gelatin in PBS at 37°C for 2 h. The test sera and 100 μl of a 0.2% BSA (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 PBS (0.05% Tween 20 in PBS), bound antibody was detected with 1:5000-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 the selected LAM mimotopes, a structurally flexible linker (Gly-Gly-Gly-Ser) was added to the selected LAM mimotopes at the desired concentrations for 24 h at 37°C in a humidified chamber with 5% CO2. The reaction was stopped by the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 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, MA) were seeded with 2 × 105 to 3 × 105 J774A.1 cells or BMDD in a total volume of 500 μl 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. The cell culture supernatants were harvested from E. coli (Sigma Chemical Co.) wells or from a positive control M. tuberculosis cell culture supernatant at 72 h and 96 h, respectively. Purified MAb CS-35 (0.3 μM) was titrated with increasing concentrations of PepCS-35[7] in 200 μl of PBS at room temperature (25 ± 1°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/F0 (where F0 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/F0 values ± the standard errors of the means (SEMs) were plotted against the peptide concentration. The F/F0 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).

$$Q = 1 + \frac{(Q_0 - 1) \times ([\text{Peptide}] + [\text{Protot}])}{K_D - \sqrt{([\text{Peptide}] + [\text{Protot}])}}$$

where Q is the observed fluorescence ratio, Q0 is the maximum peptide-induced fluorescence ratio of the antibody, Peptide is the total concentration of the peptide, and Protot is the total concentration of the protein. The parameters of the fit were Q0 and KD. Curve fitting and statistical deductions were done by using Kryptol 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 μg per well) or synthetic peptides (5 μg per well) were immobilized onto a precoated Immobilon-P PVDF membrane (Millipore Corporations) 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 serum (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 Genie 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.

Anti-LAM MAb CS-35 was used for the direct binding of LAM peptide mimotopes. The dissociation constant (Kd) 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 MAb following the interaction with antibodies. 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 spectrophotometer, 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 μM) was titrated with increasing concentrations of PepCS-35[7] in 200 μl of PBS at room temperature (25 ± 1°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/F0 (where F0 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/F0 values ± the standard errors of the means (SEMs) were plotted against the peptide concentration. The F/F0 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).
immunoactive antigens on the mycobacterial cell surface. After
the third round of panning, the peptide-coding regions of ran-
domly selected phage clones were sequenced. The results (Ta-
ble 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 phase 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 eval-
uation of the reactivity of the selected phage clones (CS-35[7]-1 to 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 arbi-
trarily designated R1, R2, and R3. To confirm that the immu-
nization 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

| Clone sequence identifier | Amino acid sequence | Frequencya |
|---------------------------|--------------------|-------------|
| 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-Leu-Pro-Ille-Met-Trp | 1/9        |

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

The results (Fig. 3) showed that there was a substantial enrichment of R1 immune serum
binding phages relative to that of R1 preimmune serum bind-
ing phages at each round of panning. As expected, the R1
preimmune serum subtraction step resulted in a further in-
crease 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

Biopanning of the Ph.D.-7 library was performed with R1
rabbit serum. Three rounds of panning were performed with a
substraction step with R1 preimmune serum, which was in-
cluded 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
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preimmune serum subtraction step resulted in a further in-
crease 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 phase 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; concen-
trated 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 µl per well) and
(B) the protein A-Sepharose-purified MAb (1 µg 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 mono-
clonal antibody (1:5,000) and ABTS substrate. Color development was
monitored at 405 nm. The results are expressed as the means ± SDs
for three independent experiments.]

binding peptide sequences selected after third round of panning
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 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-

| Clone sequence identifier | Amino acid sequence | Frequency* |
|---------------------------|--------------------|------------|
| 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       |

* Number of clones with the sequence/total number of clones tested.
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. Whatever differences 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 \( P < 0.05 \) differential reactivities (difference in the absorbance [OD405] of the immune sera from that of the preimmune sera [ΔOD405]) 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 (ΔOD405) were determined. The results (Fig. 5) show that by and large all the clones were reactive to both sera, except
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 KD 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 KD was determined. The KD value thus obtained was found to be 7.55 ± 0.83 μM (SEM) (P = 1.09 × 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 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 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. 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.

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/F₀ values are means ± SEMs (indicated by the error bars) of three independent experiments. The excitation and emission were 295 and 333 nm, respectively. In the plot, F₀ is the initial fluorescence and F is the fluorescence obtained at a particular concentration of peptide.
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 BMMs 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 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 BMMs (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 BMMs (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...
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-

FIG. 8. Stimulatory effects of peptides on cytokine release. J774A.1 cells or BMMφ (2 × 10⁵ to 3 × 10⁵ 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 (white circles) and BMMφ (black circles) 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.
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*<sub>D</sub> for the interaction of the peptide representing the 7-mer mimotope with MAb CS-35 was obtained. When the *K*<sub>D</sub> value of 7.55 μM was converted to the association constant (*K*<sub>a</sub>), a value of ~1 × 10<sup>5</sup> M<sup>−1</sup> was obtained. This value is comparable to those of the binding constants for several antigen-antibody interactions (39, 56). A *K*<sub>a</sub> 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 immunoreactive 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 BMMΦ. 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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