THE JOURNAL OF IMMUNOLOGY

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This information is current as of July 1, 2014.

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Identification of Specific Proteins and Peptides in *Mycobacterium leprae* Suitable for the Selective Diagnosis of Leprosy[1]

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Diagnosis of leprosy is a major obstacle to disease control and has been compromised in the past due to the lack of specific reagents. We have used comparative genome analysis to identify genes that are specific to *Mycobacterium leprae* and tested both recombinant proteins and synthetic peptides from a subset of these for immunological reactivity. Four unique recombinant proteins (ML0008, ML0126, ML1057, and ML2567) and a panel of 58 peptides (15 and 9 mer) were tested for IFN-γ responses in PBMC from leprosy patients and contacts, tuberculosis patients, and endemic and nonendemic controls. The responses to the four recombinant proteins gave higher levels of IFN-γ production, but less specificity, than the peptides. Thirty-five peptides showed IFN-γ responses only in the paucibacillary leprosy and household contact groups, with no responses in the tuberculosis or endemic control groups. High frequencies of IFN-γ-producing CD4$^+$ and CD8$^+$ T cells specific for the 15- and 9-mer peptides were observed in the blood of a paucibacillary leprosy patient. 9-mer peptides preferentially activated CD8$^+$ T cells, while the 15-mer peptides were efficient in inducing responses in both the CD4$^+$ and CD8$^+$ T cell subsets. Four of the six 9-mer peptides tested showed promising specificity, indicating that CD8$^+$ T cell epitopes may also have diagnostic potential. Those peptides that provide specific responses in leprosy patients from an endemic setting could potentially be developed into a rapid diagnostic test for the early detection of *M. leprae* infection and epidemiological surveys of the incidence of leprosy, of which little is known.   *The Journal of Immunology,* 2005, 175: 7930–7938.

T he prevalence of leprosy worldwide has been reduced dra- matically, mainly through global coverage of multidrug therapy, from ~10 million cases in 1985 to ~514,000 registered cases in 2003. New cases remain obstinately high in many parts of the world (1–3). In the absence of appropriate tests, we know little of the global incidence of leprosy. Diagnosis of the disease is still based largely on clinical symptoms, which can be verified by confirming the presence of acid-fast bacilli in suspected lesions, an approach not widely practiced in leprosy control set- tings. The single greatest need from leprosy research remains the development of relatively simple diagnostic tools that could rap- idly detect early infection and therefore underlying disease and allow interventions before the onset of clinical symptoms.

Certain tests have been used in the past with varying degrees of success. The finding of the leprosy-specific Ag, phenolic glyco- lipid-I, has been exploited by ELISA for the presence of phenolic glycolipid-I-specific Abs (4–6), as these Abs are present in high titers in multibacillary (MB)[3] leprosy patients. However, the Ab titer is generally low or absent in paucibacillary (PB) patients. Other diagnostic tests based on cell mediated immunity against leprosy-specific protein Ags have been investigated, foremost among them the IFN-γ whole blood assay, or a simple delayed- type hypersensitivity skin test response, analogous to the purified protein derivative (PPD) test used for tuberculosis (TB) (7). Be- cause prior immunization with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and exposure to environmental mycobac- teria are hurdles that must be overcome in the development of any protein- or peptide-based diagnostic test, we were hopeful that the

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[3] Abbreviations used in this paper: MB, multibacillary; PB, paucibacillary; PPD, pu- rified protein derivative; TB, tuberculosis; BLAST, basic local alignment search tool; BLASTP, protein-protein BLAST; BCG, bacillus Calmette-Guérin; LL, lepromatous; qRT-PCR, quantitative RT-PCR; BT, borderline tuberculoid; BL, borderline lepro- matous; HC, household contact; EC, endemic control; NEC, nonendemic control; SEB, staphylococcal enterotoxin B; CFP-10, 10-kDa culture filtrate protein; ESAT-6, early secreted antigenic target, 6 kDa.

recent information provided by comparative mycobacterial genomics (8, 9) will allow the development of Ags that are highly specific for leprosy.

Through comparative analyses of these annotated genomes and comparing with sequences from environmental mycobacteria, we have selected putative open reading frames found only in the *Mycobacterium leprae* genome, with no homologues in any of the current mycobacterial databases. We have conducted preliminary studies on four of these unique Ags to determine whether any are potentially useful in the development of a specific test for the early diagnosis of leprosy. In addition to the whole recombinant proteins, peptides selected from 26 *M. leprae* open reading frames predicted as having high scores for binding to human class II HLA-DR or class I molecules were synthesized. This article characterizes the IFN-γ responses of PBMC from leprosy and TB patients, contacts, and controls toward these proteins and peptides.

Materials and Methods

Selection of M. leprae genes and peptides

Comparative bioinformatic analyses of the *M. leprae* and *Mycobacterium tuberculosis* genomes using various programs developed by Drs. N. G. Stoker and S. T. Cole were used to generate lists of *M. leprae* genes that were not present in *M. tuberculosis*, or in other mycobacteria using sequence information available at that time, and that appeared likely to be expressed genes. *M. leprae*-predicted proteins (⟨ftp://ftp.sanger.ac.uk/pub/pathogens/leprae/⟩) were compared with six-frame translations of other mycobacterial genomes, both finished (*M. tuberculosis*) and unfinished (*Mycobacterium avium* and *Mycobacterium smegmatis*), using TBLASTN from National Center for Biotechnology Information basic local alignment search tool (BLAST) (version 2.0) (10). Preliminary sequence data for *M. avium* and *M. smegmatis* were obtained online from The Institute for Genomic Research web site (⟨www.tigr.org⟩). Protein sequences were also used to search a nonredundant protein library from GenBank using protein-protein BLAST (BLASTP). Searches were run on May 17, 2001. All sequences finding a match with a value of e < 0.01 were discarded. Using the lists generated by these methods, we selected putative open reading frames to target for recombinant protein production. The selected open reading frames were screened subsequently for homologies with the genome sequences of *M. marinum* (⟨www.sanger.ac.uk/Projects/M_marinum⟩) or *M. avium* and *M. smegmatis* (⟨www.tigr.org/tdb/mdb/mdbinprotgress.html⟩). Peptide sequences (15 or 9 mer) were selected as having high scores for binding to human class II HLA-DR (DRB1*0101, DRB1*0301 (DR17), DRB1*0401 (DR4w4), and DRB1*1101) or human class I (HLA-A*0201) using the SYFPEITHI program (⟨www.bmi-heidelberg.com/syfpeithi⟩). All of the peptides were then screened for homologies to sequences in other proteins by BLASTP using a program suitable for BLAST searches for short nearly exact matches (⟨www.ncbi.nlm.nih.gov/BLAST⟩). Peptides with significant degrees of homology to genes expressed in other potential human pathogenic microorganisms to which T cell sensitization might be expected (e.g., *Streptococcus sp.*, *Haemophilus influenzae*) were excluded. The peptides used in this study were synthesized commercially (Mimotopes), with a free amino and carboxy terminus, in 1 mmol scale. Each was dissolved in endotoxin-free distilled water, sonicated, and stored at either −70°C or aliquoted and relyophilized to dryness.

Production of recombinant M. leprae proteins

The DNA sequences coding for the four novel *M. leprae* proteins (ML0008, ML0126, ML1057, and ML2567) were amplified from *M. leprae* genomic DNA using rTth DNA polymerase XL (Applied Biosystems). PCR amplifications were performed using each of the following primer sets designed to introduce *Nde*I or *Nhe*I and *Xho*I or *Hind*III sites, respectively (underlined), to the 5′ and 3′ ends of the open reading frame: for ML0008, forward primer, 5′-ACACATATGGCAACAATTAGAACGGTAA-3′ (*Nde*I), and reverse primer, 5′-ATAAGCTTTTTTGTGTATCGAAGACAGT-3′ (*Hind*III); for ML0126, forward primer, 5′-TCTCATATGAGAGCAGCCGAAGCTTCGA-3′ (*Nde*I), and reverse primer, 5′-CGTATCTCGAGAGGGTCGACCCGGGC-3′ (*Xho*I); for ML1057, forward primer, 5′-AACATATGCAGAGGAGAACGAGATGGAT-3′ (*Nde*I), and reverse primer, 5′-ATGAAGCTTGCCTAAGTGGAGCGATC-3′ (*Hind*III); and for ML2567, forward primer, 5′-AAAGCTAGCGTGAGGCCGGGCGAGTTCC-3′ (*Nhe*I), and reverse primer, 5′-GGCTAATAAGCTTGCCGCACATGCG-3′ (*Hind*III). The PCR products were digested with the restriction enzymes *Nde*I/*Nhe*I and

*Hind*III/*Xho*I and cloned into the expression vector pET 29a(+), which uses a T7 promoter-driven system to achieve high levels of recombinant protein expression with a C-terminal 6-histidine tag. The DNA sequences of recombinant clones were confirmed by automated nucleotide sequencing (model 377; Applied Biosystems) at Macromolecular Resources Laboratory, Colorado State University. The plasmids containing the four novel Ags were introduced into the *Escherichia coli* expression host BL21 (DE3) (Invitrogen Life Technologies) by transformation. Recombinant proteins were produced as soluble proteins as described previously (11). LPS content, as measured by the limulus amoebocyte lysate test (BioWhittaker), was found to be <2 ng/mg protein.

Abs and Western blot analysis

Polyclonal antisera against the recombinant proteins were raised in BALB/c mice and used in immunoblotting as described previously (11). Native *M. leprae* cytosol, membrane, and cell wall fractions were prepared as described previously (12). Proteins (1–30 μg) from each of the subcellular fractions were separated on 15% SDS-PAGE and transferred to nitrocellulose membrane as previously described (13) for probing with Abs.

Human serum samples and ELISA

Serum samples from lepromatous (LL) leprosy patients were selected from anonymous serum samples stored at the Department of Microbiology, Yonsei University College of Medicine. Approval of the use of these serum samples was given by the internal review boards at Yonsei University College of Medicine and Colorado State University and complied with federal rules for their use.

Recombinant Ags were diluted to 1.0 mg/ml PBS (pH 7.2) and were dispersed using a probe sonicator. Each Ag was then diluted further to 5 μg/ml in carbonate-bicarbonate buffer (pH 9.6), and 100 μl of the diluted Ag was added to wells of 96-well flat-bottom EIA plates and kept overnight at 4°C for coating. The ELISA to detect Abs in patient serum samples was performed as described previously (11).

Preparation of cDNA from M. leprae RNA for quantitative RT-PCR (qRT-PCR)

M. leprae strain Thai-53 was purified from infected nude mouse footpad tissue using a previously described protocol (14). The RNA was purified from 2×10^{10} bacteria or directly from the minced biopsy material using a single-tube homogenization/RNA extraction protocol (15). Total *M. leprae* RNA was converted to cDNA using the Advantage cDNA Polymerase Mix and Advantage RT-for-PCR kit (BD Clontech) with random hexamers according to the manufacturer's recommendations. qRT-PCR assays for six *M. leprae* genes, including *groES* (ML0380), encoding 10-kDa GroES; *bfrA* (ML2038c), encoding bacterioferritin; and ML0008c, ML0126, ML1057, and ML2567, encoding four unique hypothetical unknown proteins, were developed using TaqMan technology. Primers and probes were designed for qRT-PCR assays by acquiring gene sequences from the *M. leprae* genome database (⟨www.Sanger.ac.uk/Projects/M. leprae⟩) and using Primer Express software (Applied Biosystems). All genes were amplified from the same *M. leprae* cDNA template. The qRT-PCR data was analyzed using the relative quantitation method for gene expression based on the appropriate standard curve and expressed as mean and SD.

Study population for cell-mediated assay

For the analysis of IFN-γ responses of *M. leprae* recombinant proteins and peptides, a total of 60 volunteer subjects was enrolled, 10 in each group. Untreated PB (10 borderline tuberculoid (BT) patients), and MB (3 LL and 7 borderline LL (BL) patients) leprosy patients, and household contacts (HC) of MB patients were recruited from the Souza Araujo Outlet Unit, Oswaldo Cruz Foundation. Untreated PPD skin test positive pulmonary TB patients were recruited from the Clementino Fraga Filho Hospital, Federal University of Rio de Janeiro. Healthy individuals living in Rio de Janeiro and with an unknown history of exposure to leprosy and/or TB were included as an endemic control (EC) group, while individuals living in the state of Colorado served as the nonendemic control (NEC) group. The tests and procedures described in this work were approved by the Oswaldo Cruz Foundation Ethics Committee and the internal review board of Colorado State University.

In vitro culture of PBMC with Ags

Blood was drawn by venipuncture, heparinized, and PBMC were isolated using Lymphoprep (Pharmacia Biotech) by density gradient centrifugation, washed in PBS, and resuspended in AIMV medium (Invitrogen Life Technologies) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin,

and 2 mM L-glutamine (Sigma-Aldrich). PBMC from each individual were seeded at 2×10^5 cells/well in 96-well flat-bottom plates in duplicate (BD Biosciences) and stimulated in vitro with armadillo-derived *M. leprae* whole cells (20 μg/ml), individual recombinant proteins or peptides (10 μg/ml), or staphylococcal enterotoxin B (SEB; 1 μg/ml) (Sigma-Aldrich). Cultures were incubated at 37°C in a humidified 5% CO$_2$ atmosphere. Supernatants were harvested at day 5 of incubation and stored immediately at −70°C until performing the cytokine ELISA. IFN-γ levels were assayed in duplicate using the IFN-γ DuoSet ELISA Development System kit (R&D Systems) with a detection limit of 125 pg/ml.

CD69 expression and IFN-γ intracellular staining

PBMC were seeded (5×10^5/well) in 96-well flat-bottom microtiter plates in complete AIMV medium and were stimulated with single peptides (p51, p52, p59, p61, p65, and p69) or a pool of these peptides in the presence of costimulatory mAbs specific for CD28 and CD49d. The culture plates were incubated in a humidified 37°C incubator for a total of 6h, with the last 1 h including 10 μg/ml brefeldin A (Sigma-Aldrich). After incubation the cells were permeabilized, followed by fixation with 1% paraformaldehyde in PBS. The cells were washed with wash buffer (0.5% BSA and 0.1% NaN$_3$ in PBS) stained with 2 μl of combined anti-IFN-γ FITC, anti-CD69 PE, and anti-CD4 PerCP-Cy5.5 (BD Pharmingen) or 20 μl of combined anti-IFN-γ FITC, anti-CD69 PE, anti-CD8 PerCP-Cy5.5, and anti-CD3 APC (BD Pharmingen). Control tubes were stained as above but with isotype-matched mAbs.

Flow cytometric analysis

Four-color flow cytometric acquisition and analysis were performed on a FACSCalibur flow cytometer using BD CellQuest Pro software (BD Biosciences). Forty thousand CD4$^+$ T cells and 10,000 CD8$^+$ T cells were collected typically. The cells were gated on CD4$^+$ and CD3+/CD8$^+$ T cells vs side-scattering to isolate CD4$^+$ T and CD8$^+$ T cells, then gated on forward light-scattering vs side-scattering to analyze the lymphocyte population. Data were analyzed and displayed as dot plots of IFN-γ vs CD69 fluorescence. A negative region for each cell population was defined with isotype control Abs. CaliBRITE beads (BD Pharmingen) were used for setting photomultiplier tube voltages, fluorescence compensation, and instrument sensitivity before use.

Statistical analysis

ANOVA (Statistica, version 6.0; Statsoft) was performed to determine differences in IFN-γ levels induced by each Ag among PB patients, MB patients, HC individuals, TB patients, and EC individuals. In this analysis, the cutoff was defined as twice the value of the detection limit (250 pg/ml). This analysis was followed by the Tukey test used as a post hoc test for two-group comparisons. Results between groups were considered significant when p was ≤0.05.

Results

Identification of homologies of peptides and proteins in other mycobacterial databases

The criteria for selecting many of the peptides from unique hypothetical unknown proteins and other proteins of interest from *M. leprae* was performed in early 2002. Since this study was initiated, sequences from other mycobacterial genome projects have become available, including the completed sequence of *M. bovis* (16) and the nearly completed or preliminary sequences of the genomes of *M. avium*, *M. marinum*, *M. smegmatis*, *M. ulcerans*, and *M. paratuberculosis*. The most recent search (August 25, 2005) using the BLASTP program for short nearly exact matches identified additional homologies within the 58 peptides that were selected. Peptide p55 from ML2567 (9-mer) showed 100% identity with an 8-mer from another gene in *M. leprae*, ML0314, a putative esterase, although these proteins are not otherwise related. Peptides p62 from ML0678c (83% identity of a 12-mer in gene MAP3814c), p86 and p87 from ML0840c (80% identity of a 10-mer peptide in gene MAP2122 and 66% identity of a 15-mer in the same gene, respectively), and p88 and p89 from ML1189c (84% identity of a 13-mer in gene MAP2344 and 80% identity of a 15-mer in the same gene, respectively) all had relatively high identities with peptides from *M. avium paratuberculosis* strain K10. Peptide p69

Table I. M. leprae *peptides predicted to have high binding scores to HLA MHC molecules and tested for IFN-γ responses in this study*[a]

Peptide	ID/Gene	Location	Sequence
p37	ML0008c	aa 1–15	MATIRTVRNLKLCNP
p38*	ML0008c	aa 21–35	TRLLTVVVKQRSKAF
p39*	ML0008c	aa 24–38	LTVVVKQRSKAFRPS
p40*	ML0008c	aa 107–121	RVSYGSECRSGNCLR
p41	ML0008c	aa 2–10	ATIRTVRNL
p42*	ML0126	aa 250–264	LDDFLSLQRSISPNS
p43	ML0126	aa 90–104	ATHYFEMTSDAFFAD
p44*	ML0126	aa 251–265	DDFLSLQRSISPNSY
p45	ML0126	aa 65–79	DVKIAVDPACKLSAR
p46	ML0394c	aa 117–131	HLILRITPGIDLREL
p47*	ML0394c	aa 73–87	LNQLVSLVKQIFVQQ
p48*	ML0394c	aa 22–36	QLMYLIEITSETKAL
p49*	ML1057	aa 10–24	GRYYAEINSAKMYFG
p50	ML1057	aa 46–60	VLVLVGDEAAALEQL
p51*	ML1057	aa 54–68	AAALEQLLGQTADVA
p52*	ML1057	aa 59–67	QLLGQTADV
p53*	ML2567	aa 95–109	RRTVKILRPLPSNIT
p54*	ML2567	aa 72–86	NHAVSSDFKTRSTNT
p55	ML2567	aa 128–136	DIAARLASL
p56*	ML0308	aa 17–31	FDEYRAMFALSAMDL
p57	ML0308	aa 139–153	THAFDLVLSSHLLFT
p58	ML0308	aa 194–208	DDLLARLRADGVHGE
p59	ML0398c	aa 15–29	MLILGLLPAILPACG
p60	ML0398c	aa 288–302	AILYRKIHGQDPAER
p61*	ML0398c	aa 16–24	LILGLLPAI
p62	ML0678c	aa 31–45	PYRFHSLAAIWALSP
p63*	ML0757c	aa 6–20	GINLPKDELTAFGRK
p64	ML0757c	aa 74–88	VGAVRIVGGVRPQNF
p65*	ML1419c	aa 108–122	EAVLLRLDGTTLEVE
p66*	ML1419c	aa 137–151	QVIFRDLTTQKAAEE
p67*	ML1419c	aa 51–65	SGRVTYLNPVGVKWM
p68*	ML1419c	aa 267–275	LLEEGVIVL
p69*	ML1419c	aa 113–121	RLDGTTLEV
p70*	ML1420	aa 93–107	MQEYRGLTSHTPCCR
p71*	ML1553 *proS*	aa 180–194	LDIYTTLARDMAAIP
p72	ML1553 *proS*	aa 154–168	TIEFLWQEGHSAHIE
p73*	ML1829	aa 108–122	DAEWLKLTSLGLRPR
p74*	ML1915	aa 89–104	VKAVVDDVNAILLTGR
p75*	ML2177c	aa 169–183	LQPYRLLRGGDSEYW
p76*	ML2498	aa 75–89	VGKVQGLLARLLTLP
p77*	ML2703 *trxB*	aa 331–335	DSTDTTDWSTAMTDA
p78	ML0411	aa 264–278	GLDSIISSASASLLT
p79	ML0098 *fbpC*	aa 84–98	GNAMTTLGGRGISVV
p80*	ML0126	aa 125–139	YGQVVRDVENTLRYL
p81*	ML0126	aa 180–194	GDVWKSIVHLRSTRH
p82*	ML0394c	aa 141–155	KKRLTLLSGAMARRA
p83*	ML0394c	aa 97–111	DATFQVIFSQHVHLS
p84	ML0638	aa 1–15	MIDYNNVFGAGVVAA
p85	ML0638	aa 49–63	NYEVSPIFARWPRNR
p86*	ML0840c	aa 287–301	VYLYNYLLAETSHVL
p87	ML0840c	aa 251–265	YRYYRLIATTDAASP
p88*	ML1189c	aa 55–69	DDIWRTLASAVITGN
p89	ML1189c	aa 10–24	FDSFDRILKARSPEA
p90	ML2347	aa 20–34	KENVIIVAAKISWTL
p91*	ML2347	aa 301–315	LATVQYDDRRRFTKE
p92*	ML2452c	aa 28–42	LQAYSNLFGRTSAMQ
p93	ML2591 *mce1C*	aa 62–76	GMDVGKVEALKIDGD
p94	ML2596	aa 165–179	WASVALLTAAGVSLG

[a] The information on all of the genes, sequences, and class was obtained from ⟨www.genolist.pasteur.fr/Leproma/.⟩ Peptides that elicited responses in only the leprosy patient or contact groups and not in the TB or EC groups appear with an asterisk; bold indicates the peptide is from a hypothetical unknown protein (class VI).

from ML1419c (9-mer) showed 100% identity with an 8-mer from *Streptomyces coelicolor*. Peptides p79 from ML0098 (*fbpC*) and p93 from ML2591 (*mce1C*), which had homologues in *M. tuberculosis* and were selected to examine cross-reactive responses in TB individuals, also showed high identity with peptide sequences in *M. avium paratuberculosis* strain K10 (86% identity with a 15-mer in *fbcC1* and 93% identity with a 15-mer in gene MAP3606).

A search of homologies with the four hypothetical unknown genes in this study found that although ML0008, ML1057, and ML2567 did not have orthologues in *M. tuberculosis* or other currently available mycobacterial databases, a gene with 69% identity to ML0126 was identified in the *M. ulcerans* genome (⟨http://geno-pole.pasteur.fr/Mulc/Burulist.html⟩). The six 15-mer peptides from ML0126 (p42–p45, p80, and p81) that were tested in our study showed between 67 and 87% identity with predicted sequences in *M. ulcerans*. As more sequences are added to mycobacterial databases, other orthologues and homologues to the class VI hypothetical unknown proteins of *M. leprae* may be identified.

IFN-γ responses of PBMC toward recombinant proteins and peptides

The list of the synthetic peptides and the genes from which they were selected is shown in Table I. PBMC from leprosy patient groups (PB and MB), leprosy HC, TB patient, healthy EC, and healthy NEC groups were cultured with recombinant proteins, peptides and control Ags, and IFN-γ production was measured on day 5 of culture. The results for the IFN-γ responses to four of the recombinant proteins and controls are shown in Fig. 1, while representative responses to a subset of the 58 peptides is shown in Fig. 2. Responses to all of the leprosy peptides and protein Ags in the NEC group were uniformly negative, while IFN-γ responses were below or, in a few cases, just above the detection limit, in all unstimulated cultures (medium alone) for all groups. All individuals responded well when their cells were cultured in the presence of the superantigen SEB. The responses to the four recombinant proteins were generally higher than with individual peptides, with more responses in the EC and TB groups (ML0008, 6 EC and 2 TB responders; ML0126, 9 EC and 2 TB responders; ML1057, 10 EC and 1 TB responders; ML2567, 7 EC and no TB responders) (Fig. 1). The responses of PBMC from Brazilian leprosy patients and contact individuals toward most of the peptides showed remarkable specificity, stimulating IFN-γ production in 60–100% of the 10 BT patients (PB group) and 50–100% of the 10 subjects in the HC group. Between 1–4 of 10 individuals in the MB group responded to certain peptides. Thirty-five of the peptides showed no positive responses in the TB or EC groups, and good responses in the PB and HC groups (peptides indicated by an asterisk in Table I), while 23 of the peptides showed one or more heightened IFN-γ responses in either the TB or EC groups. A few of the peptides that elicited the most frequent responses in the EC or TB groups were

those that had homologues in *M. tuberculosis*, such as peptides p79 (*fbpD* homologue Rv3803c, 80% identity), p93 (*mce1C* homologue Rv0171, 73% identity), and p72 (*proS* homologue Rv2845c, 27% identity). Other peptides that induced responses in the EC or TB groups had very low or no discernible homology with their counterpart in *M. tuberculosis*, such as peptides p57 and p60. A few of the peptides from several hypothetical unknowns, with no homologue to any gene in mycobacterial databases, induced unexpected responses in the EC or TB groups, such as p37 from ML0008. It may be that cross-reactive epitopes within these peptides will be revealed as more mycobacterial sequences are added to the database.

An analysis of the original data indicated that certain individuals in the PB group responded well to the majority of the peptides, while other individuals responded weakly or not at all to many of the peptides; those in the TB and EC group were nonresponsive to almost all of the peptides (Fig. 3). The best responders in these groups also reacted well to the *M. leprae* whole cell Ag preparation. Generally, members of the HC group responded as well as, or better than, individuals in the PB group, which was expected because most HC, although having been exposed to the disease for varying amounts of time, are healthy but immune, so their T cell responses to *M. leprae* Ags are quite robust. In summary, the responses in the leprosy patients, contacts, and controls to the majority of peptides were quite specific, showing none of the cross-reactivity that has been reported with whole recombinant proteins that have low homology (17, 18).

Four of the six 9-mers showed good potential specificity. Three of these six 9-mer peptides were contained within one of the tested 15-mer peptides: p52 within p51 (ML1057), p61 within p59 (ML0398c), and p69 within p65 (ML1419c). For the pairs of peptides from ML1057 and ML1419c, both the longer 15-mer peptide and the shorter 9-mer peptide were specific, inducing good responses in the PB and HC groups, and no response in the TB, EC, or NEC groups; for the peptides from ML0398c, the shorter 9-mer peptide appeared more specific than the longer 15-mer peptide. Overall, 31 of 52 of the 15-mer peptides gave promising specificity, while 4 of 6 of the 9-mer peptides did. This indicates that shorter 9-mer peptides, which would be expected to induce Ag-specific CD8 activation, as well as longer peptides that would be mainly recognized by CD4$^+$ T cells, have diagnostic potential.
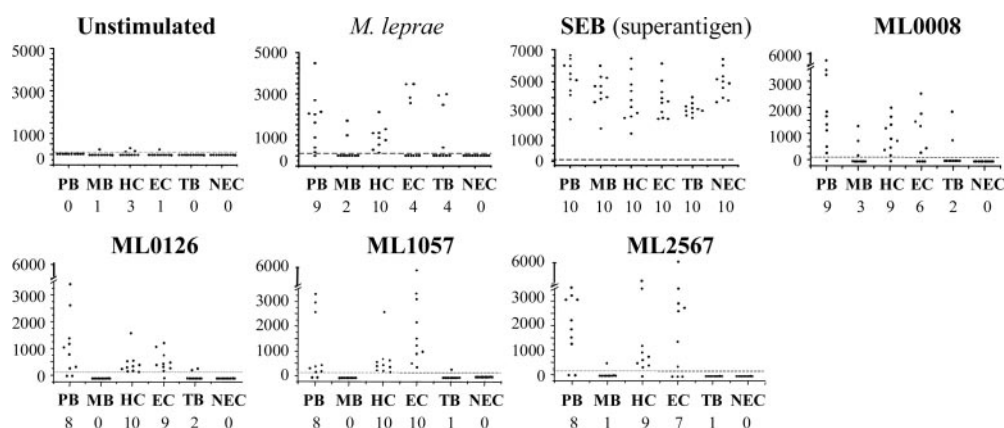
FIGURE 1. IFN-γ responses of PBMC from PB and MB leprosy patients, HC, TB patients, and healthy EC and NEC to the four *M. leprae* recombinant hypothetical unknown proteins (ML0008, ML0126, ML1057, and ML2567), *M. leprae* whole cells, and the superantigen SEB. Medium alone (unstimulated) represents IFN-γ responses in the absence of antigenic stimulus. The dashed line represents the cutoff of 125 pg/ml IFN-γ measured in the culture supernatants. The number of total responders appears below each group.
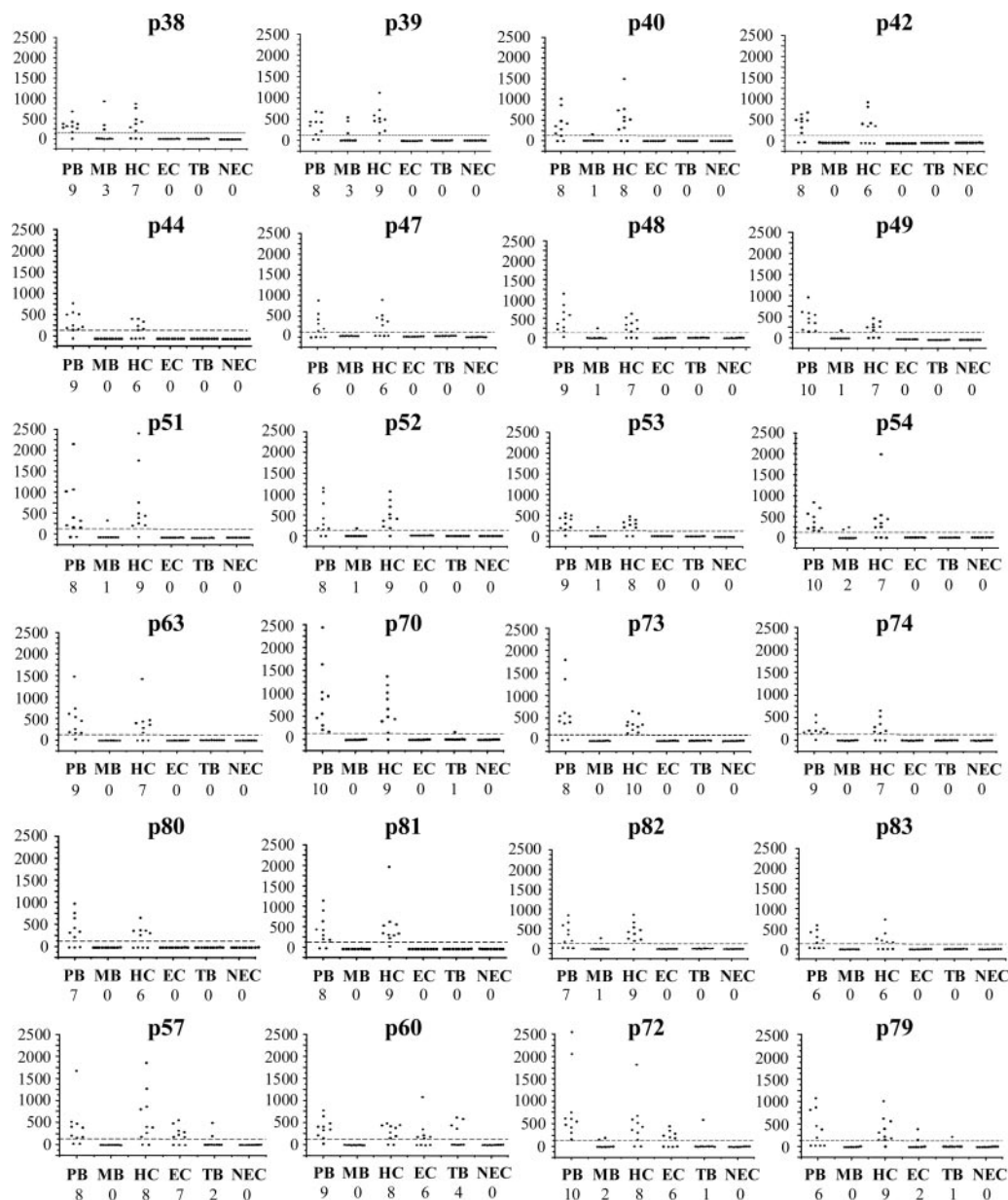
FIGURE 2. Representative IFN-γ responses of PBMC from PB and MB leprosy patients, HC, TB patients, and healthy EC and NEC to peptides comprising a subset of the 58 peptides tested. Twenty peptides induced responses in the PB and HC groups, with no responses in either the TB or EC groups. Peptides p57, p60, p72, and p79 were peptides that induced responses in multiple individuals in the TB and EC groups in addition to responses in the PB and HC groups. The dashed line represents the cutoff of 125 pg/ml IFN-γ measured in the culture supernatants. The number of total responders appears below each group.

*Frequency of CD69+ and IFN-γ-producing CD4+ and CD8+ T cells to 9- and 15-mer peptides*

To define the phenotype of T cells responding to the 15- and 9-mer peptides, the frequencies of peptide-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells present in the blood from a BT patient were analyzed by flow cytometry. The results show elevated frequencies of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells that were induced after stimulating with either single 15- or 9-mer peptides or a mixture of the same peptides. When 9-mers and 9-mer-containing 15-mers were compared, the 9-mer peptides preferentially activated $CD8^+$ T cells, while the 15-mer peptides induced responses in both $CD4^+$ and $CD8^+$ T cell subsets (Fig. 4). This observation is in agreement with previous data, indicating that 15-mer peptides are effective for the activation of both $CD4^+$ and $CD8^+$ T cells (19).

Statistical analysis of responses of the groups to individual peptides

The levels of IFN-γ induced by individual peptides in the different groups of subjects were compared for statistical significance using ANOVA. In addition to the ANOVA test, the Tukey test was used as a post hoc test for evaluating significant differences between two groups. When individually analyzed, three types of responses could be distinguished, represented in Fig. 5 by peptides p67, p69, and p75. The plots display the mean values for each group, and the 0.95 confidence intervals were adjusted using least squares for ANOVA modeling. IFN-γ responses to p69 were significantly higher in HC ($p = 0.02$) and in PB ($p = 0.01$) in comparison with those seen in MB and control groups (TB and EC). Twenty-nine of the 35 peptides that showed no positive responses in the TB and EC groups induced this type of response and can be potentially
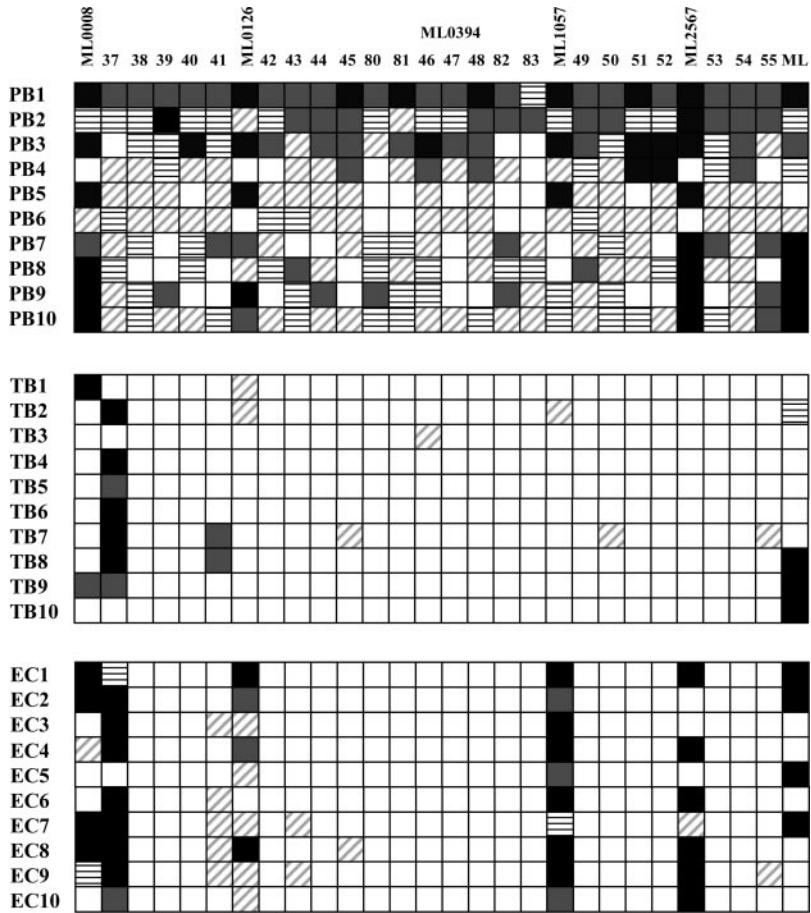
FIGURE 3. Individual IFN-γ responses from the PB, TB, and EC groups against the four recombinant proteins and their respective peptides and the peptides from ML0394 and against whole *M. leprae*. ▤ indicates the relative amounts of IFN-γ detected; □, <125 pg/ml (less than detection limit cutoff); ▨, 125–299 pg/ml; ▤, 300–499 pg/ml; dark gray, 500–999 pg/ml; ■, >1000 pg/ml.

used to detect both HC and PB. Peptide p75, in contrast, induced significantly higher levels of IFN-γ in HC as compared with PB ($p = 0.04$) and MB ($p = 0.001$) and control groups ($p = 0.001$). Three other peptides (p68, p91, and p92) induced the same type of response and can potentially be used to distinguish healthy infected individuals. Finally, IFN-γ responses to p67 were significantly higher in PB ($p = 0.01$) as compared with HC ($p = 0.04$) and to those seen in MB ($p = 0.01$) and control groups ($p = 0.01$) and show diagnostic potential for PB leprosy.

*Expression and serological reactivity toward hypothetical unknown proteins*

Polyclonal antisera raised against the four hypothetical unknown proteins were used to probe the native subcellular fractions for the presence of these Ags. However, within the limits of sensitivity of this approach, there was no detectable signal found for any of these proteins by immunoblot (data not shown). We then designed probes and primers for the four hypothetical unknowns and two well-expressed proteins, ML0380 (*groES*) and ML2038c (*bfrA*), in an attempt to understand the relationship between the cDNA transcript levels and protein expression. ML0008c (the lowest expressed transcript, assigned the arbitrary value of 1.00) was used as the calibrator to obtain the relative expression values of the other genes; therefore, values are expressed as fold increase expression over ML0008c. The results demonstrated that of the six gene transcripts analyzed using mouse footpad *M. leprae* mRNA, the most highly represented cDNA was that of ML0380, followed by ML2038c (Table II), data which are consistent with their relative abundance as expressed proteins. The ML0126 transcript level was ∼2-fold higher than ML0008c, but caution should be used in in-

terpreting whether this result indicates whether there is a similar fold increase in relative protein expression, as the value was so low. Unknown proteins ML2567 and ML1057 showed considerably higher values at 5.20 and 8.83, respectively. The same relative expression of these genes was observed from cDNA obtained from both nude mouse footpad derived *M. leprae* and from *M. leprae* obtained from human biopsy material (data not shown).

The reactivity of leprosy patient sera toward the hypothetical proteins was examined by ELISA. The majority of LL patient sera reacted well to both ML0050 (10-kDa culture filtrate protein (CFP-10)) and fusion proteins (CFP-10/early secreted antigenic target, 6 kDa (ESAT-6) and 10 kDa/ESAT-6), whereas the responses to the hypothetical proteins showed much lower responses overall (Fig. 6). This result probably reflects the very low levels of expression of these proteins as detected by Western blot analysis. Alternatively, the lack of Ab recognition of these *E. coli*-produced recombinant proteins could be the result of improper folding, as three of them contain multiple cysteines that could potentially be involved in disulfide linkages (ML0008, ML0126, and ML2567). Nevertheless, there were more positive responses against ML2567 and ML1057 than with ML0008 or ML0126, which is consistent with the higher gene transcript levels found with these proteins.
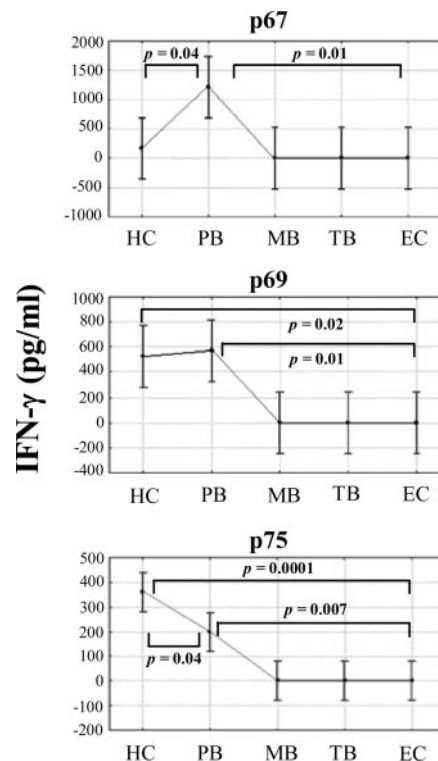
Discussion

Diagnostic tests based on cell-mediated immune responses against *M. leprae* have been studied because it was determined that intradermal inoculation of a heat-killed extract of *M. leprae*, called lepromin, induced characteristic responses in individuals infected with the disease (20, 21). A number of laboratories have examined the antigenic makeup of the *M. leprae* ever since a reliable source

A

B
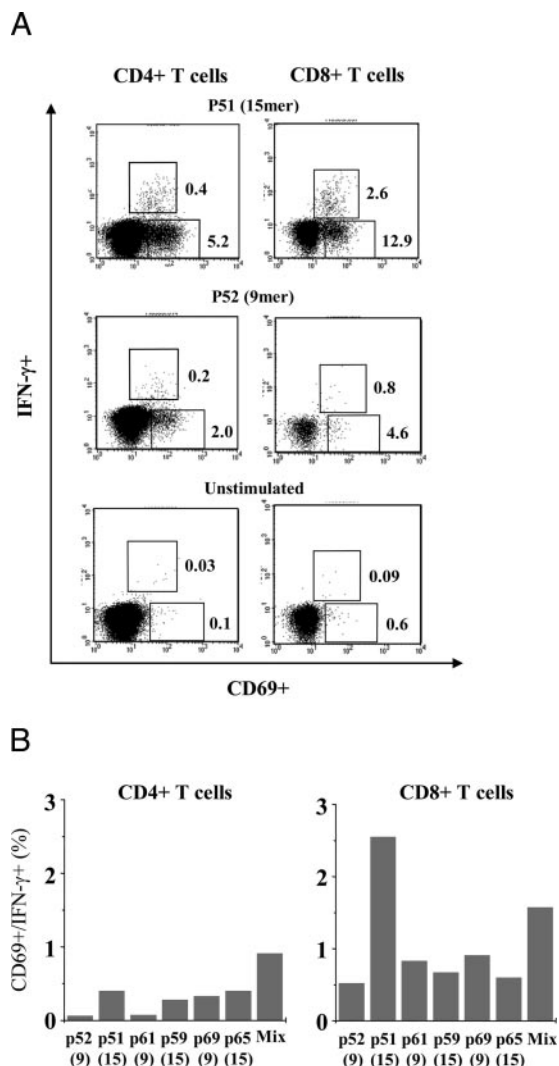
FIGURE 4. Peripheral ex vivo frequencies of peptide-specific IFN-γ producing CD4+ and CD8+ T cells in a BT leprosy patient. PBMC were stimulated with single peptides (p51, p52, p59, p61, p65, and p69 at 10μg/ml) and a mixture of peptides (10 μg/ml each). Six hours later, the cells were stained using the FastImmune CD4 (anti-IFN-γ FITC, anti-CD69 PE, and anti-CD4 PerCp-Cy5.5) or CD8 (anti-IFN-γ FITC, anti-CD69 PE, anti-CD3-APC, and anti-CD8 PerCp-Cy5.5) intracellular cytokine detection kits. The analysis was performed on lymphocytes after gating on CD4+ T cells (40,000 events) and CD8+ T cells (10,000 events) in a FACSCalibur flow cytometer. *A*, Dot plots show the frequency of p51- and p52-responding IFN-γ-producing CD69+/CD4+ T and CD69+/CD8+ T cells. *B*, The histogram bars show the frequencies of IFN-γ-producing CD69+/CD4+ and CD69+/CD8+ T cells responding to the 9-mer peptides p52, p61, and p69 and to the 9-mer-containing 15-mer peptides p51, p59, and p65, respectively, and to a mixture of these six peptides.

FIGURE 5. ANOVA of IFN-γ levels induced by *M. leprae* synthetic peptides. The vertical bars denote 0.95 confidence intervals. Values of *p* were as follows: p67, *p* = 0.00003; p69, *p* = 0.00003; and p75, *p* = 0.00008. The Tukey test was used as a post hoc test for evaluating the significant difference between groups, and the *p* values ≤0.05 are indicated in the graphics. PB, PB leprosy patients; MB, MB leprosy patients; TB, TB patients; and EC, healthy EC.

have very high homology. Although these and many other common Ags have been shown to be highly immunogenic, their inclusion in the native crude subcellular fractions would certainly lead to cross-reactive immune responses. Thus, the incorporation of native subcellular fractions in a skin test or in vitro IFN-γ assay with patient whole blood or PBMC may not have the requisite specificity, similar to experiences with PPD as a predictor of exposure to TB in countries where TB and leprosy are endemic and exposure to nonpathogenic mycobacteria in the soil and water is high.

To circumvent this problem, our laboratory has begun to focus on Ags that have either less homology to their counterparts in *M. tuberculosis* or have been shown to be unique to the *M. leprae* genome. IFN-γ secretion in the culture supernatants was used as the readout for the T cell response because it has been shown that

of purified whole cells first became available through the growth of *M. leprae* in the armadillo in the 1970s (22, 23). We have characterized a number of the dominant Ags found in each of the three main subcellular fractions (cytosol, membrane and soluble cell wall proteins), which include the major cytosolic proteins, major membrane proteins, and major cell wall Ags (12, 24, 25). A recent analysis of the cytosolic and membrane proteins by two-dimensional gels, with a characterization of tryptic-digested protein spots by tandem mass spectrometry, MALDI-TOF, and N-terminal sequencing, revealed a total of 43 proteins that had not been identified previously (26). Despite doubling the number of known proteins, virtually all of them have counterparts in *M. tuberculosis* that

Table II. *Relative transcript expression levels of four hypothetical unknown proteins and two well-expressed proteins (ML0380 (10-kDa GroES) and ML2038c (bacterioferritin)) as determined by TaqMan RT-PCR analysis of nude mouse footpad-derived* M. leprae[a]

Transcript/Gene Name	Relative Expression
ML0008c	1.00
ML0126	2.08
ML2567	5.20
ML1057	8.83
ML2038c *bfrA*	17.52
ML0380 *groES*	36.68

[a] ML0008c was assigned the value of 1.00, while the relative expression of the other transcripts was calculated based on this reading.
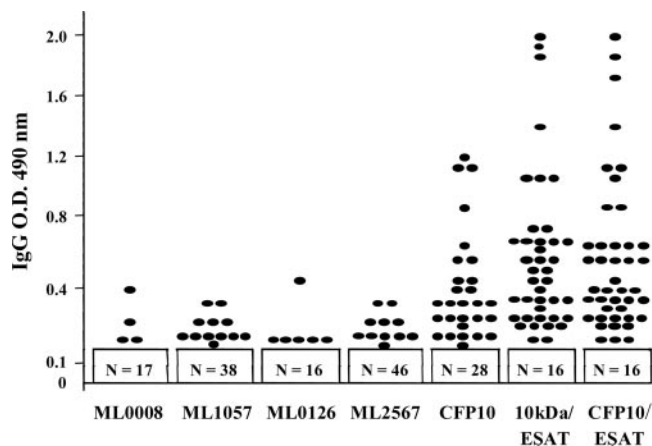
FIGURE 6. Reactivity of leprosy patient sera by ELISA to the novel *M. leprae* recombinant proteins ML0008, ML1057, ML0126, and ML2567 compared with other proteins known to be expressed at higher levels ESAT-6 (ML0049), CFP-10 (ML0050), and 10 kDa (ML0380). Two of the proteins tested are fusion proteins consisting of 10 kDa/ESAT-6 and CFP-10/ESAT-6. N values in boxes represent the number of individual sera that gave negative OD readings.

protective immunity to mycobacterial infection requires the specific activation of T cells secreting type 1 cytokines, such as IFN-γ (27, 28). Initially, we predicted that due to the comparatively low amounts of message and lack of positive results in detecting these hypothetical unknown proteins by Western blot analysis, T cell responses would be correspondingly low. The surprising finding is that not only are there positive responses in the majority of the PB and HC groups to all four of the whole recombinant proteins tested but that there are also positive responses with almost all of the peptides from these same proteins. There were higher average amounts of IFN-γ produced in all groups to the whole proteins, but there were also many more positive responses in the EC and TB groups, indicating that, as with the case with ESAT-6 and CFP-10, there may be cross-reactive epitopes present. However, the responses to the majority of the peptides (35 of 58, or 60%) were remarkably specific, inducing positive responses only in the PB and HC groups and no responses (or in rare cases, just above the detection limit of 125 pg/ml IFN-γ) in any of the control groups. An interesting observation was the diagnostic potential of a number of the peptides (p67, p68, p75, p91, and p92) in terms of their capacity to discriminate among the known *M. leprae*-infected individuals (PB patients) and exposed but healthy individuals who have not yet developed any symptoms of the disease (HC group). It is possible that the detection of responses in the HC group and not PB or MB individuals are the result of latency-associated Ags, similar to that which has been found in healthy individuals infected with *M. tuberculosis*. Further study of these peptides using a much larger group size will be required to determine whether this relationship holds.

There have been a number of recent studies that have evaluated T cell responses of TB patients and BCG-vaccinated individuals toward recombinant proteins and peptides derived from genes found in regions of difference (designated regions RD1 to RD16), regions of the genome that are present in virulent strains of *M. tuberculosis* and *M. bovis*, but which are absent in all or most of the BCG vaccine strains (29, 30). These studies have identified ESAT-6 and CFP-10, two of the most intensely studied Ags, both of which have shown great potential for TB diagnosis (31, 32), and for the detection of latent TB infection (33). The use of combinations of peptides from these two Ags provided a high level of

sensitivity and specificity in IFN-γ T cell assays (34, 35). A number of other potential TB-specific peptide Ag candidates have been identified from these RD region genes, and some of these have been examined for sensitivity and specificity by themselves and in combination with ESAT-6 and CFP-10 proteins (36–38). Although some peptides from the RD1 PPE protein Rv3873 were shown to be cross-reactive in nature due to the high homology with peptides from conserved regions in other members of the PPE family, others were shown to be very specific (36). Similarly, the findings in our present study that there were more frequent responses in the EC and TB groups to the whole recombinant proteins is perhaps an indication of possible cross-reactive responses toward peptides that show homology to as yet unidentified peptides from other mycobacteria, even though three of four whole proteins examined were supposedly "unique" to *M. leprae*. Other studies have shown fewer problems with cross-reactivity using peptides compared with using the whole protein, so this may be one way to avoid undesirable cross-reactivity by selecting only those peptides that stimulate responses in the targeted group (38, 39).

Our observation that IFN-γ responses were detected to both shorter 9-mer peptides and to longer 15-mer peptides may also have important implications. The fact that we detected *M. leprae*-specific responses to both shorter 9-mer peptide and 15-mer peptides that contained the shorter 9-mer peptides indicate that such 15-mer peptides might show greater sensitivity, as both Ag-specific CD4$^+$ and CD8$^+$ T cells are activated by such sequences. The recent observation that, contrasting with CD8$^+$ T cells, the CD4$^+$ T cell population requires the presence of Ag for proliferation even in the course of an inflammatory response, supports the evaluation of the frequency of CD4$^+$ T cells specific for *M. leprae* Ags as a tool for detecting active and latent *M. leprae* infection (40). Although it was harder to select suitable 9-mer peptides for testing, as shorter sequences tended to show greater homologies to sequences in other genes, the specific responses detected here to four of the six 9-mer peptides tested indicates that Ag-specific CD8$^+$ T cell responses are also present and may have diagnostic potential. A recent BLASTP search for short nearly exact matches with our panel of peptides revealed homologies with sequences in recently added mycobacterial databases. Although this new information appears to affect a minority of peptides, further testing will be required to determine whether some of these candidates should be eliminated from the pool due to problems with cross-reactivity.

To design a diagnostic test that is both highly sensitive and specific for leprosy, it is likely that multiple Ags or peptides will have to be incorporated as a mixture or as a polyprotein because it has been shown that the use of multiple Ags increases the frequency of responses in infected individuals. Factors that influence the response to individual components of an Ag mixture include the genetic makeup (due to MHC polymorphisms) of the individual and whether a given peptide can be presented by that person's MHC molecules; the immune status of the individual, who may be nonresponsive due to HIV infection or disease induced anergy; and the variation of the T cell response to particular Ags during the course of infection, which is probably very important for those with tuberculoid vs LL forms of the disease. Currently, we are examining the responses of larger numbers of leprosy patients and controls with pooled peptides, in the hope to increase the overall response in the PB and HC groups, and perhaps even increase the percentage of responders in the MB group, while retaining a high level of specificity. It is important to remember that in the context of an endemic country, examining the responses in the healthy

endemic control population is the most relevant. It should be possible to combine a number of these peptides to formulate a highly sensitive and specific cell-mediated test that will allow the early and rapid diagnosis of leprosy.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

References

1. World Health Organization. 2003. Leprosy elimination campaigns: impact on case detection. *Wkly. Epidemiol. Rec.* 78: 9–16.
2. World Health Organization. 2002. Leprosy elimination campaigns. *Wkly. Epidemiol. Rec.* 77: 17–20.
3. World Health Organization. 2002. Leprosy: global situation. *Wkly. Epidemiol. Rec.* 77: 1–8.
4. Cho, S. N., S. H. Kim, R. V. Cellona, G. P. Chan, T. T. Fajardo, G. P. Walsh, and J. D. Kim. 1992. Prevalence of IgM antibodies to phenolic glycolipid I among household contacts and controls in Korea and the Philippines. *Lepr. Rev.* 63: 12–20.
5. Chin-a-Lien, R. A., W. R. Faber, M. M. van Rens, D. L. Leiker, B. Naafs, and P. R. Klatser. 1992. Follow-up of multibacillary leprosy patients using a phenolic glycolipid-I-based ELISA: do increasing ELISA-values after discontinuation of treatment indicate relapse? *Lepr. Rev.* 63: 21–27.
6. Buhrer-Sekula, S., H. L. Smits, G. C. Gussenhoven, J. Van Leeuwen, S. Amador, T. Fujiwara, P. R. Klatser, and L. Oskam. 2003. Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy. *J. Clin. Microbiol.* 41: 1991–1995.
7. Brennan, P. J. 2000. Skin test development in leprosy: progress with first-generation skin test antigens, and an approach to the second generation. *Lepr. Rev.* 71(Suppl.): S50–S54.
8. Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, et al. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393: 537–544.
9. Cole, S. T., K. Eiglmeier, J. Parkhill, K. D. James, N. R. Thomson, P. R. Wheeler, N. Honore, T. Garnier, C. Churcher, D. Harris, et al. 2001. Massive gene decay in the leprosy bacillus. *Nature* 409: 1007–1011.
10. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389–3402.
11. Spencer, J. S., H. J. Kim, A. M. Marques, M. Gonzalez-Juarrero, M. C. B. S. Lima, V. D. Vissa, R. W. Truman, M. L. Gennaro, S-N. Cho, S. T. Cole, and P. J. Brennan. 2004. Comparative analysis of B- and T-cell epitopes of *Mycobacterium leprae* and *Mycobacterium tuberculosis* culture filtrate protein 10. *Infect. Immun.* 72: 3161–3170.
12. Marques, M. A. M., S. Chitale, P. J. Brennan, and M. C. V. Pessolani. 1998. Mapping and identification of the major cell wall-associated components of *Mycobacterium leprae*. *Infect. Immun.* 66: 2625–2631.
13. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* 76: 4350–4354.
14. Truman, R. W., and J. L. Krahenbuhl. 2001. Viable *Mycobacterium leprae* as a research reagent. *Int. J. Lepr. Other Mycobact. Dis.* 69: 1–12.
15. Williams, D. L., Oby-Robinson, S., Pittman, T. L., and D. M. Scollard. 2003. Purification of *Mycobacterium leprae* RNA for gene expression analysis directly from biopsy specimens of leprosy patients. *Biotechniques* 35: 534–541.
16. Garnier, T., K. Eiglmeier, J. C. Camus, N. Medina, H. Mansoor, M. Pryor, S. Duthoy, S. Grondin, C. Lacroix, C. Monsempe, et al. 2003. The complete genome sequence of *Mycobacterium bovis*. *Proc. Natl. Acad. Sci. USA* 100: 7877–7882.
17. Geluk, A., K. E. van Meijgaarden, K. L. Franken, Y. W. Subronto, B. Wieles, S. M. Arend, E. P. Sampaio, T. de Boer, W. R. Faber, B. Naafs, and T. H. Ottenhoff. 2002. Identification and characterization of the ESAT-6 homo-

18. Geluk, A., K. E. van Meijgaarden, K. L. M. C. Franken, B. Wieles, S. M. Arend, W. R. Faber, B. Naafs, and T. H. M. Ottenhoff. 2004. Immunological cross-reactivity of the *Mycobacterium leprae* CFP-10 with its homologue in *Mycobacterium tuberculosis*. *Scand. J. Immunol.* 59: 66–70.
19. Maecker, H. T., H. S. Dunn, M. A. Suni, E. Khatamzas, C. J. Pitcher, T. Bunde, N. Persaud, W. Trigona, T. M. Fu, E. Sinclair, et al. 2001. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. *J. Immunol. Methods* 255: 27–40.
20. Mitsuda, K. 1919. On the value of a skin reaction to a suspension of leprosy nodules. *Jap. J. Der. Urol.* 19: 697–708.
21. Fernandez, J. M. M. 1940. The early reaction induced by lepromin. *Int. J. Lepr.* 8: 1–14.
22. Draper, P. 1979. Protocol 1/79: purification of *M. leprae*. In *Annex 1 of the Enlarged Steering Committee for Research on the Immunology of Leprosy (IMMLEP) Meeting, February 7–8*. World Health Organization, Geneva, p. 4.
23. Shepard, C. C., P. Draper, R. J. W. Rees, and C. Lowe. 1980. Effect of purification on the immunogenicity of *Mycobacterium leprae*. *Br. J. Exp. Path.* 61: 376–379.
24. Rivoire, B., M. C. V. Pessolani, C. M. Bozic, S. W. Hunter, S. A. Hefta, V. Mehra, and P. J. Brennan. 1994. Chemical definition, cloning, and expression of the major protein of the leprosy bacillus. *Infect. Immun.* 62: 2417–2425.
25. Hunter, S. W., B. Rivoire, V. Mehra, B. R. Bloom, and P. J. Brennan. 1990. The major native proteins of the leprosy bacillus. *J. Biol. Chem.* 265: 14065–14068.
26. Marques, M. A. M., B. J. Espinosa, E. K. X. da Silveira, M. C. V. Pessolani, A. Chapeaurouge, J. Perales, K. M. Dobos, J. T. Belisle, J. S. Spencer, and P. J. Brennan. 2004. Continued proteomic analysis of *Mycobacterium leprae* subcellular fractions. *Proteomics* 4: 2942–2953.
27. Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme. 1993. Disseminated tuberculosis infection in interferon γ gene-disrupted mice. *J. Exp. Med.* 178: 2243–2247.
28. Caruso, A. M., N. Serbina, E. Klein, K. Triebold, B. R. Bloom, and J. L. Flynn. 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-γ, yet succumb to tuberculosis. *J. Immunol.* 162: 5407–5416.
29. Mahairas, G. G., P. J. Sabo, M. J. Hickey, D. C. Singh, and C. K. Stover. 1996. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. *J. Bacteriol.* 178: 1274–1282.
30. Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane, and P. M. Small. 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* 284: 1520–1523.
31. Arend, S. M., P. Andersen, K. E. van Meijgaarden, R. L. Skjot, Y. W. Subronto, J. T. van Dissel, and T. H. Ottenhoff. 2000. Detection of active tuberculosis infection by T cell responses to early secreted antigenic target 6-kDa protein and culture filtrate protein 10. *J. Infect. Dis.* 181: 1850–1854.
32. van Pinxteren, L. A., P. Ravn, E. M. Agger, J. Pollock, and P. Andersen. 2000. Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP10. *Clin. Diagn. Lab. Immunol.* 7: 155–160.
33. Ewer, K., J. Deeks, L. Alvarez, G. Bryant, S. Waller, P. Andersen, P. Monk, and A. Lalvani. 2003. Comparison of T cell-based assay with tuberculin skin test for diagnosis of *Mycobacterium tuberculosis* infection in a school tuberculosis outbreak. *Lancet* 361: 1168–1173.
34. Arend, S. M., A. Geluk, K. E. van Meijgaarden, J. T. van Dissel, M. Theisen, P. Andersen, and T. H. Ottenhoff. 2000. Antigenic equivalence of human T cell responses to *Mycobacterium tuberculosis*-specific RD1-encoded protein antigens ESAT-6 and culture filtrate protein 10 and to mixtures of synthetic peptides. *Infect. Immun.* 68: 3314–3321.
35. 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.
36. Okkels, L. M., I. Brock, F. Follmann, E. M. Agger, S. M. Arend, T. H. M. Ottenhoff, F. Oftung, I. Rosendrands, and P. Andersen. 2003. PPE protein (Rv3873) from DNA segment RD1 of *Mycobacterium tuberculosis*: strong recognition of both specific T cell epitopes and epitopes conserved within the PPE family. *Infect. Immun.* 71: 6116–6123.
37. Liu, X.-Q., D. Dosanjh, H. Varia, K. Ewer, P. Cockle, G. Pasvol, and A. Lalvani. 2004. Evaluation of T cell responses to novel RD1- and RD2-encoded *Mycobacterium tuberculosis* gene products for specific detection of human tuberculosis infection. *Infect. Immun.* 72: 2574–2581.
38. Brock, I., K. Weldingh, E. M. S. Leyten, S. M. Arend, P. Ravn, and P. Andersen. 2004. Specific T cell epitopes for immunoassay-based diagnosis of *Mycobacterium tuberculosis* infection. *J. Clin. Microbiol.* 42: 2379–2387.
39. Cockle, P. J., S. V. Gordon, A. Lalvani, B. M. Buddle, R. G. Hewinson, and H. M. Vordermeier. 2002. Identification of novel *Mycobacterium tuberculosis* antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. *Infect. Immun.* 70: 6996–7003.
40. Obst, R., H. M. van Santen, D. Mathis, and C. Benoist. 2005. Antigen persistence is required throughout the expansion phase of a CD4$^+$ T cell response. *J. Exp. Med.* 201: 1555–1565.