March 2009

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CLINICAL AND VACCINE IMMUNOLOGY, Mar. 2009, p. 352–359 Vol. 16, No. 3
1556-6811/09/$08.00+0 doi:10.1128/CVI.00414-08

From Genome-Based In Silico Predictions to Ex Vivo Verification of Leprosy Diagnosis▽†

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Received 8 November 2008/Returned for modification 3 December 2008/Accepted 23 December 2008

The detection of hundreds of thousands of new cases of leprosy every year suggests that transmission of *Mycobacterium leprae* infection still continues. Unfortunately, tools for identification of asymptomatic disease and/or early-stage *M. leprae* infection (likely sources of transmission) are lacking. The recent identification of *M. leprae*-unique genes has allowed the analysis of human T-cell responses to novel *M. leprae* antigens. Antigens with the most-promising diagnostic potential were tested for their ability to induce cytokine secretion by using peripheral blood mononuclear cells from leprosy patients and controls in five different areas where leprosy is endemic; 246 individuals from Brazil, Nepal, Bangladesh, Pakistan, and Ethiopia were analyzed for gamma interferon responses to five recombinant proteins (ML1989, ML1990, ML2283, ML2346, and ML2567) and 22 synthetic peptides. Of these, the *M. leprae*-unique protein ML1989 was the most frequently recognized and ML2283 the most specific for *M. leprae* infection/exposure, as only a limited number of tuberculosis patients responded to this antigen. However, all proteins were recognized by a significant number of controls in areas of endemicity. T-cell responses correlated with in vitro response to *M. leprae*, suggesting that healthy controls in areas where leprosy is endemic are exposed to *M. leprae*. Importantly, 50% of the healthy household contacts and 59% of the controls in areas of endemicity had no detectable immunoglobulin M antibodies to *M. leprae*-specific PGL-I but responded in T-cell assays to ≥1 *M. leprae* protein. T-cell responses specific for leprosy patients and healthy household contacts were observed for ML2283- and ML0126-derived peptides, indicating that *M. leprae* peptides hold potential as diagnostic tools. Future work should concentrate on the development of a sensitive and field-friendly assay and identification of additional peptides and proteins that can induce *M. leprae*-specific T-cell responses.

Leprosy is a curable infectious disease caused by *Mycobacterium leprae* involving cutaneous tissue and peripheral nerves and causing skin lesions, nerve degeneration, anesthesia, and deformities. Important advances in antimycobacterial therapy in the 1980s were the basis of the effort by the World Health Organization to eliminate leprosy as a public health problem (i.e., to achieve a global prevalence of <1/10,000) by the year 2000. The leprosy elimination program has had a massive effect on the registered number of cases, which fell to 212,802 worldwide at the beginning of 2008 (1). In addition, a reported year-end prevalence below 1 per 10,000 in 2007 was obtained in all but three countries with populations of >1 million (Brazil, Nepal, and East Timor). The global number of new cases detected has continued to decrease dramatically in the last 5 years at an average rate of nearly 20% per year (1). Notwithstanding these numbers, hundreds of thousands of new cases of leprosy are still detected every year (254,525 worldwide in 2007), and pockets of high endemicity, where leprosy remains a public health problem, still occur in Angola, Brazil, the Central African Republic, India, Madagascar, Nepal, and the United Republic of Tanzania. This demonstrates that active transmission is occurring in the face of an antibiotic-based leprosy elimination strategy, and this transmission is thought to be caused by the continuing reservoir of *M. leprae*-infected contacts and persons with subclinical leprosy. Furthermore, a population survey in Bangladesh showed that even in the presence of a strong leprosy control program, the actual number of leprosy cases was about five times higher than the registered number of cases (17). Therefore, timely detection and prompt multidrug treatment are of utmost importance. However, diagnosis of leprosy is classically based on clinical manifestations

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† Supplemental material for this article may be found at http://cvi.asm.org/.

▽ Published ahead of print on 28 January 2009.

as well as labor-intensive and time-consuming laboratory or histological evaluation. The scarcity of symptoms in early disease and the lack of bacteria in paucibacillary (PB) patients contribute to the difficulty of diagnosis. Tools for diagnosis of asymptomatic *M. leprae* infection are not available yet, nor is it possible to predict disease development in exposed individuals. While the existence of high-titer immunoglobulin M (IgM) antibodies to phenolic glycolipid-I (PGL-I) has allowed the development of user-friendly kit-based tests, these are applicable largely to multibacillary (MB) leprosy patients (9) and have little relevance to those with PB or asymptomatic leprosy who show vigorous cellular rather than humoral immune responses (18). Thus, in order to allow informed decision making on who needs treatment at a preclinical stage, new tests that detect *M. leprae* infection and/or measure biomarkers that predict disease development in infected individuals are required.

Cellular tests have in the past relied on the use of complex and usually incompletely defined mixtures of *M. leprae* components (4) and have limited value due to their inherent high cross-reactivity with other mycobacteria, which is particularly problematic in countries with high incidence rates of tuberculosis (TB), routine *Mycobacterium bovis* BCG vaccination, and high levels of exposure to environmental mycobacteria. In our attempts to develop simple assays based on cell-mediated immune (CMI) responses particularly for identification of asymptomatic leprosy, we were encouraged by the recent development of two commercially available gamma interferon (IFN-γ) release assays for specific diagnosis of *M. tuberculosis* infection (11, 19) that exploit antigens (ESAT-6, CFP-10, and TB7.7) that are selectively expressed by *M. tuberculosis* and deleted in nonvirulent BCG strains and most other nontuberculous mycobacteria. However, the *M. leprae* homologues of ESAT-6 and CFP-10 (ML0049 and ML0050, respectively) were recognized well by T cells from *M. tuberculosis*-infected individuals, despite limited amino acid sequence homology (36% and 40%, respectively), thereby limiting the diagnostic potential of ESAT-6 and CFP-10 in areas of leprosy endemicity with high prevalences of TB (15, 16).

Through comparative analysis of annotated mycobacterial genomes, several investigators selected putative open reading frames that were found only in the *M. leprae* genome and lacked homologues in any of the (myco)bacterial databases available at that time. Further bioinformatic analyses of these *M. leprae*-unique sequences identified several (hypothetical) antigens that were tested for their ability to induce in vitro T-cell responses in *M. leprae*-infected individuals (2, 3, 13, 14, 20). Two initial studies that provided a basis for the currently described study were performed with a Brazilian population (Rio de Janeiro, Brazil), using *M. leprae*-unique proteins and peptides arising from the above-described approach (13, 20). Together, these studies identified several proteins and peptides with the potential to identify *M. leprae*-infected subjects.

T-cell responses are HLA restricted, which may pose a problem for the global applicability of diagnostic T-cell-based assays in genetically diverse populations. A previous study using *M. leprae*-derived peptides showed considerable variation in peptide reactivity at different sites (8). Thus, in order to estimate the potential of the peptides for detecting *M. leprae*-specific T-cell immunity in the context of genetically different backgrounds, we selected the most promising peptides (n = 22) and proteins (n = 5) from the previous studies in Brazil, four other countries of leprosy endemicity in Asia (Nepal, Bangladesh, and Pakistan) and Africa (Ethiopia), and an additional site in west central Brazil (Goiás State).

Once identified, *M. leprae* antigens that provide specific immune responses in leprosy patients and exposed individuals at all five sites of endemicity could be used to develop a rapid diagnostic test for early detection of leprosy. Such a test could be used in field studies to estimate how many individuals living in areas of endemicity have been infected with *M. leprae* and to identify those in high-risk groups who require treatment or prophylaxis.

MATERIALS AND METHODS

General procedure of the study. Five laboratories, situated in areas where leprosy is endemic, were involved in this study: Tropical Pathology and Public Health Institute, Federal University of Goiás, Goiânia, Brazil; Mycobacterial Research Laboratory, Anandaban Hospital, Anandaban, Nepal; International Center for Diarrheal Disease Research Bangladesh, Dhaka, Bangladesh; Aga Khan University, Karachi, Pakistan; and Armauer Hansen Research Institute, Addis Ababa, Ethiopia. To ensure reproducibility of data throughout the study at each site, all experiments carried out by different laboratories involved were performed according to standard operating procedures and each site was provided with identical reagents.

Production and testing of *M. leprae* recombinant proteins. *M. leprae* candidate genes were amplified by PCR from genomic DNA of *M. leprae* and cloned using the Gateway technology platform (Invitrogen, Carlsbad, CA) with a pDEST17 expression vector containing an N-terminal histidine tag (Invitrogen, Carlsbad, CA) (12). Sequencing was performed on selected clones to confirm the identities of all cloned DNA fragments. Recombinant proteins were overexpressed in *Escherichia coli* BL21(DE3) and purified as previously described to remove possibly present endotoxin (12). Each purified *M. leprae* protein was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Coomassie brilliant blue staining and Western blotting with an anti-His antibody (Invitrogen, Carlsbad, CA) to confirm size and purity. Endotoxin contents were below 50 IU/mg recombinant protein, as tested using a Limulus amebocyte lysate assay (Cambrex, East Rutherford, NJ). All recombinant proteins were tested to exclude antigen-nonspecific T-cell stimulation and cellular toxicity potentially induced by 6-day incubation with protein (as estimated by increased or decreased responses to medium or phytohemagglutinin [PHA], respectively), using IFN-γ release assays with peripheral blood mononuclear cells (PBMC) of BCG-negative, Mantoux skin test-negative, healthy Dutch donors recruited at Leiden University Medical Center (LUMC), The Netherlands. None of these controls had experienced any known prior contact with leprosy or TB patients (see Fig. S1 in the supplemental material).

***M. leprae* whole-cell sonicate.** Irradiated, armadillo-derived *M. leprae* whole cells were probe sonicated with a Sanyo sonicator to >95% breakage (Colorado State University, Fort Collins, CO, through NIH/NIAID Leprosy Contract N01-AI-25469).

Synthetic peptides. Sequences of *M. leprae* peptides were selected on the basis of data obtained in previous studies (2, 14, 20). Peptides were synthesized commercially (Mimotopes, San Diego, CA) with free amino and carboxy termini at a 1-mmol scale. Each peptide was dissolved in endotoxin-free distilled water, sonicated, aliquoted, and relyophilized to dryness.

Study subjects. Ethical approval of the study protocol was obtained through the appropriate local ethics committees. Written, informed consent was obtained from all individuals before venipuncture. At each site, 50 human immunodeficiency virus-negative individuals were recruited: 10 BL/LL leprosy patients, 10 BT/TT leprosy patients, 10 healthy household contacts (HHCs) of BL/LL patients, 10 healthy individuals from the same area of endemicity (the EC group), and 10 smear-positive pulmonary TB patients. In Ethiopia, only six HHCs were recruited. Leprosy patients were recruited between September 2006 and March 2007 and treated with chemotherapy for less than 3 months, with no signs of leprosy reactions. Leprosy was diagnosed on the basis of clinical, bacteriological, and histological observations and classified on the basis of a skin biopsy, which was evaluated by qualified personnel using the methods of Ridley and Jopling (19a). Leprosy patient recruitment was performed at the following institutes: Centro de Referência em Diagnóstico e Terapêutica/Reference Center for Diagnosis and Treatment, Goiânia, Brazil; Anandaban Hospital, Anandaban, Ne-

pal; Leprosy Control Institute & Hospital, Dhaka, Bangladesh; Marie Adelaide Leprosy Center Saddar, Karachi, Pakistan; and ALERT Hospital, Addis Ababa, Ethiopia.

HHCs were defined as adults who had been living in the same house as a BL/LL index patient for at least the preceding 6 months. EC individuals were assessed for the absence of signs and symptoms of TB and leprosy. Staff members working in the leprosy centers or clinics were excluded as EC individuals. The TB patients were required to have been on chemotherapy for at least 3 months to enable some recovery of T-cell function. For all subjects, the presence or absence of a BCG scar was recorded.

Lymphocyte stimulation tests. Venous blood samples were obtained from study participants in heparinized tubes and PBMC isolated by Ficoll density centrifugation. PBMC (2×10^6 cells/ml) were plated in triplicate cultures in 96-well round-bottom plates (Costar Corporation, Cambridge, MA) in 200 μl/well of adoptive immunotherapy medium (AIM-V; Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). Synthetic peptides, recombinant protein, *M. leprae* whole-cell sonicate, or purified protein derivative of *M. tuberculosis* (Mycos, Loveland, CO) was added to give a final concentration of 10 μg/ml. As a positive-control stimulus, a concentration of 1 μg/ml PHA (Sigma, St. Louis, MO) was used. After 6 days of culture at 37°C at 5% CO_2 and 90% relative humidity, 75-μl supernatants were removed from each well, and triplicates were pooled and frozen in aliquots at −20°C until further analysis.

IFN-γ ELISA. IFN-γ levels were determined by an enzyme-linked immunosorbent assay (ELISA; U-CyTech, Utrecht, The Netherlands) (14). The cutoff value for defining positive responses was set beforehand at 100 pg/ml. The assay sensitivity level was 40 pg/ml. Values for unstimulated cell cultures were typically <20 pg/ml. Lyophilized supernatant of PHA cultures of PBMC from an anonymous buffycoat (LUMC, The Netherlands) was provided to all five sites as a reference positive-control supernatant.

PGL-I ELISA. IgM antibodies against *M. leprae* PGL-I were detected with natural disaccharide of PGL-I linked to bovine serum albumin (0.01 ng/well; provided through NIH/NIAID Leprosy Contract N01-AI-25469) as previously described (5). Serum dilutions (100 μl/well; 1:300) were incubated at 37°C for 90 min in flat-bottomed microtiter plates (Nunc) coated with natural disaccharide of PGL-I linked to bovine serum albumin. After a wash, diluted enzyme-linked secondary antibody solution (100 μl/well) was added to all wells and incubated at 37°C for 30 min. After another wash, diluted TMB (3,3′,5,5′-tetramethylbenzidine) solution (100 μl/well) was added to all wells and incubated in the dark for 15 min at room temperature. The reaction was stopped by adding 100 μl/well 0.5 N H_2SO_4. Absorbance was determined at a wavelength of 450 nm. Samples with net optical densities at 450 nm above 0.56 were considered positive. ELISA performance was monitored using a positive and a negative control serum sample on each plate.

Statistical analysis. Differences in IFN-γ levels between test groups were analyzed with the two-tailed Mann-Whitney U test for nonparametric distribution, using GraphPad Prism (version 4). *P* values were corrected for multiple comparisons. The statistical significance level used was *P* values of <0.05.

RESULTS

T-cell recognition of *M. leprae* antigens in areas where leprosy is endemic. In order to develop tools that can be used to detect *M. leprae*-specific T-cell immunity in the context of genetically different backgrounds, a study population of 246 individuals was recruited at five institutes in areas where leprosy is endemic (Goiânia, Brazil; Anandaban, Nepal; Dhaka, Bangladesh; Karachi, Pakistan; and Addis Ababa, Ethiopia). At each site, 10 individuals from five test groups (MB, PB, HHC, EC, and TB) were targeted (Table 1). Throughout this study IFN-γ was used as a readout for T-cell responses directed against *M. leprae* antigens, as it is a stable and robust Th1 cytokine. Before IFN-γ analysis of test samples, the performances of the selected IFN-γ assay were compared for the five sites of endemicity by using identical batches of human IFN-γ standard. This showed that all five laboratories obtained overlapping IFN-γ standard curves with similar ranges of sensitivity (data not shown).

TABLE 1. Participating study sites and study populations

Site	Prevalence[a]	Category	BI (mean)	Sex ratio[b]	Mean age (yr)
Goiás State (Brazil)	4.19	MB	2.07	6/4	47
		PB	0	4/6	40
		HHC	−[c]	4/6	43
		EC	−	4/6	27
		TB	−	5/5	43
Anandaban (Nepal)	1.56	MB	2.7	9/1	42
		PB	0.08	7/3	35
		HHC	−	5/5	33
		EC	−	6/4	20
		TB	−	6/4	25
Dhaka (Bangladesh)	2.45	MB	1.60	7/3	34
		PB	0.30[d]	7/3	38
		HHC	−	7/3	38
		EC	−	7/3	31
		TB	−	7/3	29
Karachi (Pakistan)	0.39	MB	2.4	7/3	41
		PB	0	4/6	32
		HHC	−	4/6	39
		EC	−	6/4	30
		TB	−	4/6	28
Addis Ababa (Ethiopia)	0.60	MB	2.35	7/3	35
		PB	0.15[e]		8/2
		HHC	−	6/0	37
		EC	−	6/4	20
		TB	−	4/6	24

[a] Prevalence per 10,000 individuals.
[b] Male/female ratio.
[c] −, not applicable.
[d] The positive bacterial index (BI) was caused by one individual with a BI of 3.
[e] The positive bacterial index (BI) was caused by one individual with a BI of 1.5.

In order to identify antigens that induce *M. leprae*-specific T-cell responses in vivo, all individuals were analyzed for central memory T-cell responses against 5 recombinant proteins that had been identified as *M. leprae* unique (ML1989, ML1990, ML2283, and ML2567 [13] and ML2346 [10]) and 22 *M. leprae* peptides (Table 2) (2, 20) in a 6-day assay using isolated PBMC. In general, IFN-γ production in response to the control stimuli PHA, *M. leprae* (Table 3), and purified protein derivative (data not shown) was as predicted according to the subject group at all five sites. IFN-γ responses in unstimulated control samples were absent or low. Individuals with high values for in vitro unstimulated medium (>100 pg/ml) were excluded from the study and replaced, as were individuals lacking responses against PHA. IFN-γ data obtained at each of the five test sites of leprosy endemicity showed that all five recombinant *M. leprae* proteins induced IFN-γ responses, although with various degrees of specificity and interindividual differences (Fig. 1). Overall, the frequencies of responders to the proteins were highest in Nepal and lowest in Brazil (see Fig. S2 in the supplemental material).

Since the amounts of IFN-γ obtained at all five sites for PHA and *M. leprae* whole-cell sonicates were mostly within similar ranges (Table 3), IFN-γ production in response to each recombinant *M. leprae* protein was analyzed by combining individuals of all sites per test group (Fig. 1). These cumulative

TABLE 2. Amino acid sequences of synthetic *M. leprae* peptides

Peptide	Amino acid sequence	Gene annotation
p43	STNYVNLSTIGIKLKI	ML1989
p40	VGFVNAWSLLFYPPQGWESP	ML1990
p19	PLARELYRKIDCYSEEDLVE	ML2283
p20	DCYSEEDLVEIAAKSLRATI	ML2283
p92	LQAYSNLFGRTSAMQ	ML2452c
p70	MQEYRGLTSHTPCCR	ML1420
p51	AAALEQLLGQTADVA	ML1057
p73	DAEWLKLTSLGLRPR	ML1829
p63	GINLPKDELTAFGRK	ML0757c
p86	VYLYNYLLAETSHVL	ML0840c
p88	DDIWRTLASAVITGN	ML1189c
p48	QLMYLIEITSETKAL	ML0394c
p81	GDVWKSIVHLRSTRH	ML0126
p40	RVSYGSECRSGNCLR	ML0008c
p54	NHAVSSDFKTRSTNT	ML2567
p74	VKAVVDDVNAILLTGR	ML1915
p69	RLDGTTLEV	ML1419c
p56	FDEYRAMFALSAMDL	ML0308
p85	NYEVSPIFARWPRNR	ML0638
p49	GRYYAEINSAKMYFG	ML1057
p1	SRGFDEYRAMFALSAMDLNATI	ML0308
p2	SLLMQEYRGLTSHTP	ML1420

data showed that in all test groups, ML1989 was recognized (i.e., producing >100 pg/ml IFN-γ) most frequently, ranging from 30% responders in the BL/LL group and 46% in the HHC group to 56% in the BT/TT group and even 70% in the EC group. Responses to ML2283 were observed least frequently, as only three TB patients and nine BL/LL patients recognized this protein.

Although BT/TT patients, HHCs, and, to a lesser extent, BL/LL patients responded well to most of the five recombinant *M. leprae* proteins, responses were not specific for these groups, as the majority of the EC group showing in vitro T-cell responses to *M. leprae* whole-cell sonicate responded equally well to these proteins (Fig. 1).

T-cell responses to the proteins corresponded with in vitro responses to *M. leprae* whole-cell sonicate, except for two *M. leprae*-negative EC individuals whose PBMC were activated by ML1989 (Fig. 1).

In contrast to IFN-γ responses induced by recombinant *M. leprae* proteins, induction of T-cell responses to the 22 *M. leprae* peptides (Table 2) was limited (Fig. 2); although each peptide was recognized at least once, the number of individuals responding significantly (≥100 pg/ml) to one peptide or more was lower than that for protein recognition (for BL/LL patients, 16%; for BT/TT patients, 20%; for HHCs, 20%; for EC individuals, 20%; and for TB patients, 22%). This confirms what we observed previously (20), namely, that peptides induce lower IFN-γ responses and that a lower number of individuals respond to peptides. No peptide responses were detected in any individuals from Brazil. ML1420 p70, ML0394 p48, and ML0308 p56 were recognized most frequently by PBMC of *M. leprae*-exposed individuals but were also recognized by those of TB patients ($n = 6$, $n = 6$, and $n = 7$, respectively) and EC individuals ($n = 3$, $n = 2$, and $n = 3$, respectively). Overall, peptide recognition per se was not limited to *M. leprae*-infected patients or HHCs, as the percentages of individuals responding to ≥1 *M. leprae* peptide were similar among both EC individuals and TB patients. However, individuals responding to the peptides also responded to *M. leprae* whole-cell sonicate. Three *M. leprae*-unique peptides, ML2283 p19 (14), ML2283 p20 (14), and ML0126 p81 (20), were specific in all five populations, as they were recognized only by T cells from subjects in the BL/LL, BT/TT, and HHC groups, not by any EC individuals or TB patients.

Added value of T-cell responses to *M. leprae*-specific anti-PGL-I IgM antibodies. All individuals were tested for *M. leprae*-specific IgM antibodies to PGL-I. Anti-PGL-I IgM antibodies were present in 46/50 BL/LL patients, in 19/50 BT/TT patients, in 6/46 HHCs, in 3/50 EC individuals, and in 13/50 TB patients (Table 4) (optical densities at 450 nm of >0.56; range, 0.200 to 2.819).

In order to analyze whether CMI responses directed against *M. leprae* antigens may provide any added value in detecting *M. leprae* exposure/infection in comparison to currently available assays that measure *M. leprae*-specific humoral immunity, T-cell reactivities to the five *M. leprae* proteins versus humoral immune responses against *M. leprae* were analyzed (Table 4 and Fig. 3). In the HHC group, 39/46 individuals did not have detectable levels of anti-PGL-I IgM antibodies. Interestingly, 23/39 (59%) of these exposed individuals responded to one or more of the five proteins unique to *M. leprae* (Fig. 3). In the EC group, 47/50 individuals were seronegative for antibodies to *M. leprae*-specific PGL-I, and 68% (32/47) of these individuals showed T-cell responses to one or more *M. leprae* antigens.

DISCUSSION

Leprosy and its associated disabilities will be with us well into the future, as recognized in the World Health Organization's global strategy for 2006 to 2010 (1). Clearly, the methods

TABLE 3. Mean IFN-γ production levels in response to control stimuli

Group	Mean (pg/ml) for indicated stimulus[a]									
	M. leprae					PHA				
	A	B	C	D	E	A	B	C	D	E
BL/LL	781	314	435	59	748	5,553	3,387	2,407	637	5,221
BT/TT	3,014	1,882	750	953	1,799	4,940	5,331	3,087	1,826	4,814
HHC	2,848	903	1,897	1,199	2,240	5,081	4,608	4,658	745	3,181
EC	1,305	2,186	1,524	2,165	2,774	4,900	4,946	2,883	879	7,325
TB	68	1,778	248	444	1,781	5,523	4,996	1,732	705	5,032

[a] Values shown were calculated from single values corrected for background responses. Sites: A, Brazil; B, Nepal; C, Bangladesh; D, Karachi; and E, Ethiopia.

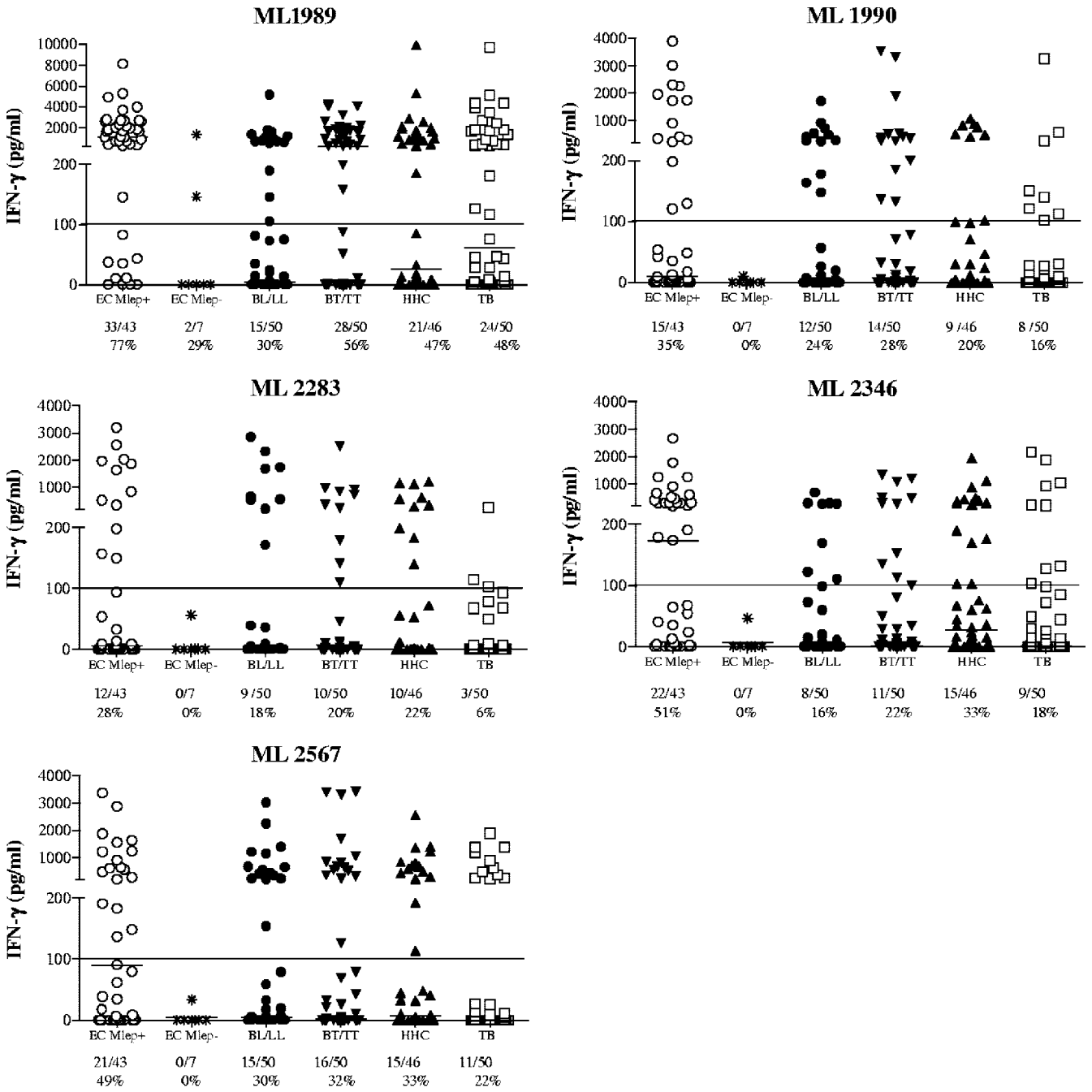

FIG. 1. IFN-γ production by PBMC induced in response to five recombinant *M. leprae* proteins (ML1989, ML1990, ML2283, ML2346, and ML2567) derived from six test groups: healthy EC individuals with (○; EC Mlep+; $n = 43$) or without (*; EC Mlep−; $n = 7$) in vitro T-cell responses to *M. leprae* whole-cell sonicate, (borderline) lepromatous leprosy patients (●; BL/LL; $n = 50$), (borderline) tuberculoid leprosy patients (▼; BT/TT; $n = 50$), HHCs (▲; $n = 46$), and TB patients (□; $n = 50$). For each test group, data from five sites where leprosy is endemic (Brazil, Nepal, Bangladesh, Pakistan, and Ethiopia) are combined. All values are corrected for medium values. Median values per test group are indicated by short horizontal lines. For each test group, the number of IFN-γ responders (>100 pg/ml, as indicated by the horizontal line) versus the total number of individuals in the group and the percentage is indicated below the *x* axis.

and knowledge available to date have not been sufficient to eliminate leprosy. Transmission continues because tools for early, preclinical diagnosis of *M. leprae* infection, which is likely to be a major source of unidentified transmission, are lacking. Thus, the design of better tools for detection of preclinical *M. leprae* infection, which would allow introduction of multidrug therapy at an early stage, has been an important goal in leprosy research since the beginning of the 21st century.

The successful use of the *M. tuberculosis*-specific peptides ESAT-6 (Rv3875), CFP-10 (Rv3874), and TB7.7 (Rv2654) for TB diagnostics among humans (11) supported our belief in the possibilities for using peptides in CMI response-based diag-

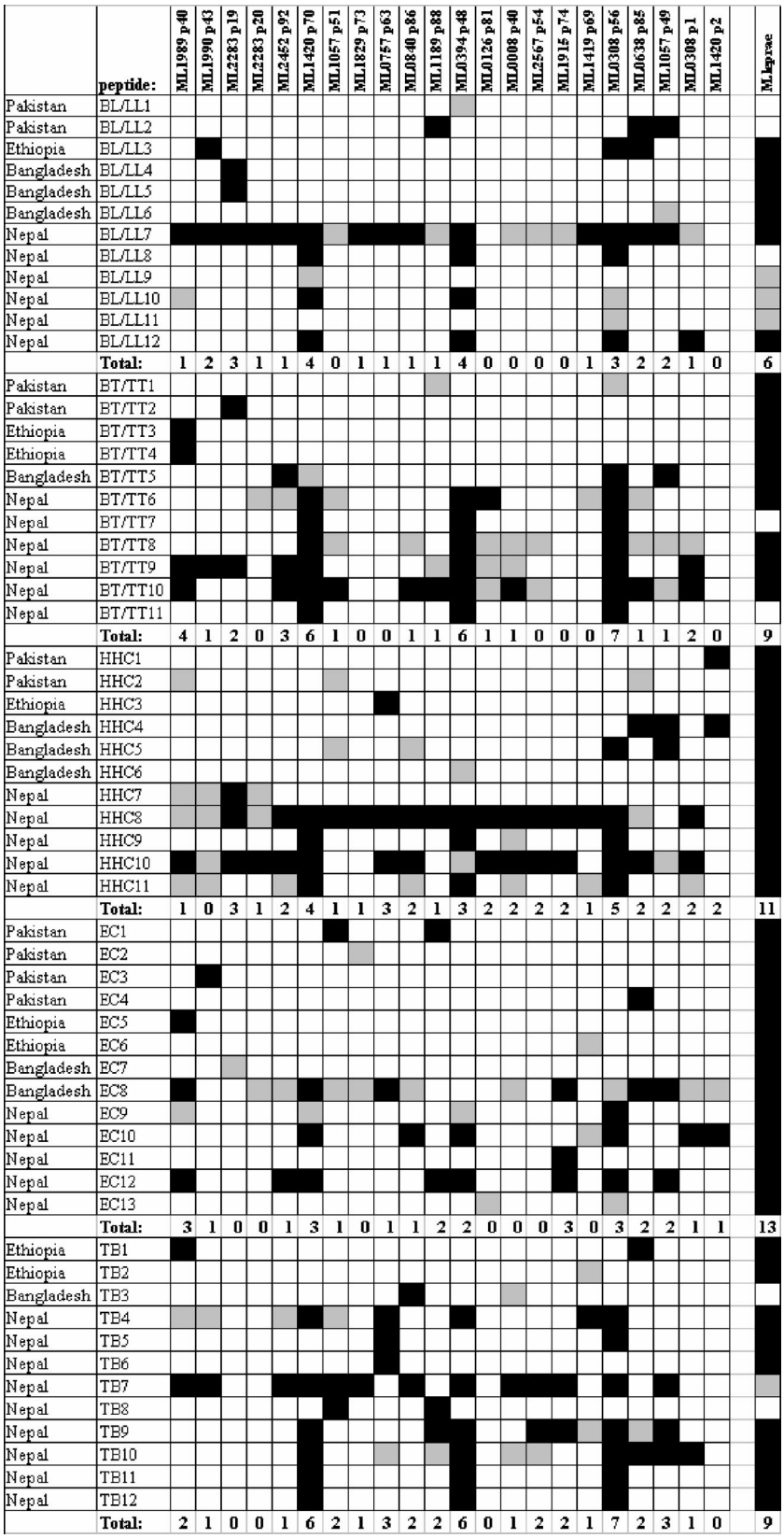

FIG. 2. IFN-γ production corrected for medium values induced in response to 22 synthetic *M. leprae* peptides (Table 2) by 6-day incubation of PBMC derived from MB leprosy patients ($n = 50$), PB leprosy patients ($n = 50$), HHCs ($n = 46$), healthy controls in areas of endemicity (EC group), or TB patients ($n = 50$). Black squares indicate IFN-γ production of >100 pg/ml; gray squares indicate IFN-γ production between 50 and 100 pg/ml; white squares indicate IFN-γ production <50 pg/ml. Numbers in each test group indicate the total number of individuals per group responding with >100 pg/ml to a certain peptide.

TABLE 4. Numbers of individuals with *M. leprae*-specific anti-PGL-I IgM antibodies

Group	No. of positive individuals/total no. of individuals (%) for indicated site[a]					
	A	B	C	D	E	Total
BL/LL	8/10	8/10	10/10	10/10	10/10	46/50 (92)
BT/TT	0/10	5/10	4/10	4/10	6/10	19/50 (38)
HHC	1/10	3/10	0/10	1/10	2/6	7/46 (15)
EC	0/10	1/10	1/10	1/10	0/10	3/50 (6)
TB	1/10	3/10	1/10	4/10	4/10	13/50 (26)

[a] Sites: A, Brazil; B, Nepal; C, Bangladesh; D, Karachi; and E, Ethiopia.

nostic tests for leprosy. The availability of the genome sequence of *M. leprae* (6), together with new techniques in bioinformatics, has thus enabled us to identify *M. leprae*-unique candidate proteins (2, 13, 20), as well as peptides (20) or combinations of peptides (14), that can be applied to detect *M. leprae*-specific T-cell responses.

In earlier studies situated in Brazil (14, 20), *M. leprae* proteins induced higher levels of IFN-γ, but T-cell responses to peptides were found to be more specific. Therefore, both *M. leprae* proteins and peptides that had shown promising specificity in these studies were analyzed in the current multicenter study, which used samples from Brazil, Nepal, Bangladesh, Pakistan, and Ethiopia.

The results of our study show that all proteins were indeed recognized strongly by PBMC of BT/TT patients and HHCs, with ML2283 being the most specific protein, as it was not frequently recognized in TB patients (Fig. 1). However, the majority of the EC group responded as well to the *M. leprae* proteins and peptides (Fig. 1 and 2) as did the individuals known to have been exposed to or infected by *M. leprae*, despite the fact that all the antigens had been selected on the basis of their unique sequences in *M. leprae*. In addition, T-cell responses against *M. leprae* antigens were observed in TB patients. Since *M. leprae*-positive EC individuals and TB patients were also responding to *M. leprae* whole-cell sonicate and none of the antigens induced any IFN-γ in healthy controls derived from countries of nonendemicity (see Fig. S1 in the supplemental material), the possibility remains that the response against *M. leprae*-unique antigens is caused by previous exposure of EC individuals and TB patients to *M. leprae*. This would then indicate that exposure to/infection by *M. leprae* may be occurring in this population at much higher rates than previously thought (17). Importantly, three *M. leprae*-unique peptides, ML2283 p19 (14), ML2283 p20 (14), and ML0126 p81 (20), were specific in all five areas of endemicity, as these peptides were recognized only in BL/LL patients, BT/TT patients, and HHCs but to a much lesser extent (50 to 100 pg/ml) in EC individuals or not at all in TB patients. Since HLA restriction allows a relatively low number of individuals to respond to these peptides, additional *M. leprae* peptides will have to be analyzed in order for a combination of peptides that detects T-cell responses to *M. leprae* in a specific fashion to be obtained. Furthermore, the variability of T-cell responses in cultures stimulated with peptides at the five different test sites (Fig. 2) highlighted the necessity of testing *M. leprae* peptides in different populations in order to design diagnostic tools that are applicable in various areas of endemicity.

Since a combination of PGL-I serology with assays based on CMI responses against *M. leprae* antigens may allow detection of most forms of leprosy (PB and MB), including preclinical leprosy, we analyzed whether these five *M. leprae* proteins represented potential added value in diagnosing early infection: T-cell responses against these proteins were detected in 59% of *M. leprae*-exposed HHCs that did not have antibodies to PGL-I (Fig. 3), indicating a serologically undetected but potentially *M. leprae*-infected group.

The development of a sensitive, specific, and field- and user-friendly test (7) which is also affordable can have a significant impact on leprosy control programs in countries of endemicity. In search of new diagnostic tools for leprosy, we have thus far found that T-cell responses to *M. leprae* proteins and peptides can be detected in a 6-day PBMC assay. In future studies, we will aim to maintain the *M. leprae* specificity of the peptides as observed here in the PBMC stimulation assay for ML2283 p19, ML2283 p20, and ML0126 p81 and, in addition, screen more *M. leprae* proteins and peptides to identify sequences that together induce CMI responses in the context of multiple HLA alleles, thereby providing coverage for diagnostics in different regions of endemicity. Since T-cell responses to *M. leprae* antigens are more sensitive in assays using PBMC than in whole-blood assays (data not shown), we will also assess whether the conditions of *M. leprae* peptide-based whole-blood assays can be optimized.

Finally, it is crucial that follow-up studies be carried out to determine whether T-cell responses as observed here are related to *M. leprae* infection or exposure and whether the presence of this CMI response is indicative of protection against leprosy or disease development.

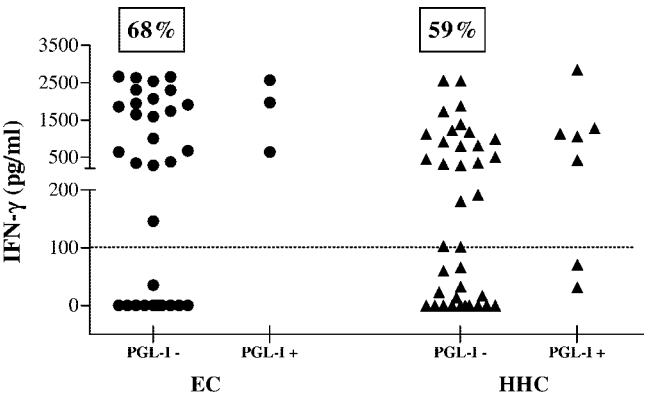

FIG. 3. Added value of in vitro T-cell responses to *M. leprae* antigens. The highest level of IFN-γ production induced by PBMC against ML1989, ML1990, ML2283, ML2346, or ML2567 is depicted for EC individuals (●) and HHCs (▲) in the context of their seropositivity for antibodies against PGL-I. The percentages of individuals lacking antibodies against PGL-I (PGL-I−) and those responding to ≥1 of the *M. leprae*-unique proteins are indicated above the figure. The total numbers of individuals that recognize a certain peptide are indicated in boxes. The threshold for positive responses is indicated by the dotted line.

ACKNOWLEDGMENTS

This study received funding from The Heiser Program for Research in Leprosy and Tuberculosis of The New York Community Trust as part of their grant to the IDEAL Consortium (http://www.ideal-leprosy.net); The Netherlands Leprosy Relief Foundation (ILEP no. 702.02.65); NIH, NIAID contract N01 AI-25469; and NIH, NIAID grant R01 AI-47197.

We thank Iris Maria Peixoto Alvim (Fiocruz), Firdaus Shahid (Aga Khan University), Mohammad Khaja Mafij Uddin (International Center for Diarrhoeal Disease Research, Bangladesh), and Jolien van der Ploeg (LUMC) for technical assistance and Tom Ottenhoff (LUMC) for stimulating discussions and critical reading of the manuscript. For patient recruitment, we are indebted to Ana Lúcia Maroclo Sousa (Goiânia, Brazil), Kapil Dev Neupane (Anandaban, Nepal), Abdul Hadi (Dhaka, Bangladesh), Ashfaq and Husna Ali (Karachi, Pakistan), and Wondimagegn Enbiale, Genet Amare, and Hassen Ali (Addis Ababa, Ethiopia).

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