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Fibronectin-Binding Antigen 85 and the 10-Kilodalton GroES-Related Heat Shock Protein Are the Predominant TH-1 Response Inducers in Leprosy Contacts

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Peripheral blood mononuclear cells from 27 healthy leprosy contacts were analyzed for lymphoproliferation and TH-1 cytokine secretion (interleukin-2 and gamma interferon) in response to heat shock proteins with molecular masses of 65, 18, and 10 kDa from *Mycobacterium leprae* and the 30-32-kDa antigen 85 (Ag 85) from *Mycobacterium bovis* BCG. Cells from 18 and 19 of 19 lepromin-positive contacts proliferated or produced TH-1 cytokines in response to the *M. leprae* 10-kDa protein and to Ag 85, respectively. Limiting-dilution analysis for two lepromin-positive contacts indicated that about one-third of *M. leprae*-reactive T cells displayed specificity to the *M. leprae* 10-kDa protein and Ag 85. The *M. leprae* 65- and 18-kDa proteins were less potent TH-1 response inducers: gamma interferon and interleukin-2 could be measured in 14 and 9 of 19 lepromin-positive contacts, respectively. In contrast, very low or undetectable proliferative and cytokine responses were found for 8 lepromin-negative contacts. Our data demonstrate that the fibronectin-binding Ag 85 and the 10-kDa GroES homolog are powerful mycobacterial TH-1 response inducers in the vast majority of lepromin-positive contacts and suggest that they might be valuable candidates for a future subunit vaccine.

TH-1-type helper T cells producing interleukin-2 (IL-2), gamma interferon (IFN-γ), and lymphotoxin are associated with acquired resistance to the intracellular pathogen *Mycobacterium leprae* (9, 31). Particularly, IFN-γ plays an essential role in protective immunity against mycobacteria, as convincingly demonstrated in genetically deleted IFN-γ and IFN-γ receptor knock-out mice (4, 5, 8, 11).

The precise antigens eliciting a TH-1 response in leprosy are not yet well defined in spite of characterization of a considerable number of molecules from *M. leprae* (34). Two groups of antigens have been reported to be particularly immunodominant. The first group consists of heat shock proteins (hsp), among which the 70-, 65-, and 18-kDa hsp, which are related, respectively, to the DnaK protein, the GroEL protein, and a low-molecular-mass stress protein from soybean, have been well documented. The second defined group consists of the secreted culture filtrate (CF) antigens, with a major component being the 30-32-kDa, fibronectin-binding antigen 85 (Ag 85). In previous studies we have demonstrated that Ag 85 from *Mycobacterium bovis* BCG CF is an immunodominant T-cell antigen and a more powerful IFN-γ inducer than the 70-, 65-, and 18-kDa hsp in tuberculoid leprosy patients and particularly in lepromin-positive healthy contacts (16, 19). Recently, the *M. leprae* 10-kDa hsp, which is homologous to the GroES gene product of *Escherichia coli* but for which a human homolog has not yet been identified, has also been described as an immunodominant antigen in leprosy, eliciting furthermore a strong delayed-type hypersensitivity reaction in guinea pigs sensitized with killed *M. leprae* (20).

On the one hand, secreted proteins may play an essential role in eliciting protective immunity against mycobacterial infections because live mycobacteria are far more effective than killed bacilli in induction of specific acquired resistance (26). On the other hand, the role of hsp in protective immunity is open for discussion. Indeed, since mycobacterial hsp are closely related to human hsp, these stress proteins might have the potential to induce autoimmune responses (10).

In *Mycobacterium tuberculosis* and *M. bovis* BCG-infected mice, IFN-γ-producing CD4 cells which are associated with protective immunity recognize culture filtrate proteins but not hsp 65 (12, 27). In leprosy infection, IFN-γ secretion against Ag 85 but not against the *M. leprae* 18- and 65-kDa proteins might be a predominant event in the early stages of the development of acquired immunity against *M. leprae* (18).

Cytotoxic T cells also play a role in antimycobacterial protection by lysing mycobacterium-infected macrophages (14). Indeed, CD4 and CD8 cytotoxic T cells have been observed in mycobacterial infections in both humans and mice (13, 24, 28), and mycobacteria can probably generate $CD4^+$ cytotoxic T cells with a TH-1-like cytokine profile (25). The aim of this study was to evaluate the potencies of fibronectin-binding Ag 85 and the 10-kDa GroES-related protein in inducing a TH-1 response in healthy leprosy contacts compared with the potencies of other intracellular hsp.

MATERIALS AND METHODS

Contacts. Twenty-seven household contacts (fifteen males and twelve females) aged 9 to 37 years and living in a rehabilitation village 70 km south of Dakar, Senegal, were tested for lepromin (Mitsuda test) reactivity. The length of contact with their leprous family members ranged from 3 to 32 years. None of these contacts had shown previous signs of clinical leprosy. Blood samples were drawn before skin testing, and isolated peripheral blood mononuclear cells (PBMC) were studied immediately after collection.

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Lepromin test. The Mitsuda reaction was examined with lepromin (4×10^6 acid-fast bacilli) provided by the World Health Organization (R. J. Rees, National Institute of Medical Research, London, United Kingdom). Induration was measured after 4 weeks (day 28), and the test was considered positive when the diameter of the papule was >3 mm.

Antigens. *M. leprae* extracted from armadillo tissue, irradiated, and lyophilized was provided by the World Health Organization (batch CD 128) and used at a final concentration of 10 μg/ml. *M. bovis* BCG IP Dakar and whole BCG (strain GL2) CF were prepared as previously described (6) and used at 5 μg/ml. Purified protein derivate from *M. tuberculosis* (PPD) (batch RT 40; Statens Serum Institute, Copenhagen, Denmark) was used at a concentration of 5 μg/ml. Ag 85 (30-32 kDa) was purified from a 2-week-old culture filtrate of *M. bovis* BCG (strain GL2) by sequential chromatography on phenyl-Sepharose and DEAE-Sephacel (both from Pharmacia, Uppsala, Sweden) as previously described (6) and used at 10 μg/ml. Purified GroEL (hsp 60) from *E. coli*, purified recombinant 65-kDa protein from *M. leprae* and *M. bovis* BCG (homologs of GroEL), recombinant 18-kDa protein from *M. leprae*, and recombinant 10-kDa protein from *M. leprae* (homologous to GroES/hsp 10) were kindly provided by J. Van Embden through the WHO/TDR/IMMLEP special program and used at 10 μg/ml.

Blood collection for sampling PBMC. Blood was collected by venipuncture with heparinized Vacutainer tubes (Becton Dickinson, Rutherford, N.J.). PBMC were isolated over a Ficoll-Hypaque gradient (density, 1.077) (LSM lymphocyte separation medium; Organon Teknika, Durham, N.C.) and suspended in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (all from Gibco) and with 10% human heat-inactivated AB serum (Institute J. Boy, Reims, France).

Assay of T-cell proliferation. The assay was carried out in round-bottom 96-microwell plates (Nunclon; Nunc, Roskilde, Denmark). Triplicate cultures containing 2×10^5 PBMC in 0.2 ml of culture medium with or without antigens were incubated for 7 days in a 5% CO_2-enriched humidified atmosphere. [^{3}H]thymidine (1 μCi per well, 2 Ci/mmol; Amersham, Les Ullis, France) was added for the last 16 h of culture. Cells were harvested onto glass fiber filters by using a semiautomated cell harvester system (Skatron, Lierbyen, Norway), and counts per minute were determined by liquid scintillation in a beta counter (1214 Rackbeta; Wallac, Turku, Finland). The results are expressed as the difference in counts per minute (Δcpm) between stimulated and control cultures. The threshold for positivity was a Δcpm of >5,000.

IFN-γ production. Cultures were done as described for the T-cell proliferation assay. Culture supernatants from triplicate cultures were harvested and pooled after 7 days and flown to Brussels on dry ice. The antiviral activity of IFN-γ was measured by a cytopathic effect reduction assay with vesicular stomatitis virus as the challenge virus on human amniotic WISH cells as described previously (16). IFN titers are expressed in international reference units per milliliter according to the international reference preparation NIH-Gg23-901-530. The detection level was 6 IU/ml, and all IFN values of >10 IU/ml were considered positive.

IL-2 assay. Culture supernatants were harvested after 2 days, and the IL-2 content was measured by using the IL-2-dependent CTLL-2 cell line as previously described (12). Results are expressed as the stimulation index (SI), which was calculated as counts per minute in culture with antigens/counts per minute in cultures without antigens. IL-2 assays were considered positive if the SI was >2. A serial dilution of a reference IL-2 preparation (500 IU/ml; Janssen Biochimica) was performed in duplicate in each assay.

Limiting-dilution analysis. Precursor frequency analysis was done as previously described by Modlin et al. (23). Briefly, T lymphocytes were isolated from PBMC by sedimentation of sheep erythrocyte rosettes. Replicate cultures (24 wells) were established in round-bottom microwell plates. T cells were cultured with or without antigen at 1,000, 2,500, 5,000, and 10,000 T lymphocytes per well in the presence of 10^4 mitomycin-treated nonrosetting PBMC as antigen-presenting cells. Cultures were incubated at 37°C in the presence of 5% CO_2. On day 6, 5 IU of recombinant IL-2 (Amersham) per ml was added to each well, and on day 9, 50% of the medium was removed and replaced with fresh medium containing 5 IU of IL-2 per ml. On day 12, [^{3}H]thymidine (1 μCi per well, 2 Ci/mmol; Amersham) was added for the last 8 h of culture. Cells were harvested onto glass fiber filters, and counts per minute determined in a liquid scintillation fluid beta counter.

RESULTS

Individual proliferative responses to purified antigens. Figure 1 shows the pattern of the proliferative response of blood mononuclear cells recovered from leprosy contacts. Cells from 19 of 19 lepromin-positive contacts proliferated in response to PPD, BCG CF, whole BCG, and *M. leprae*. PBMC from 17 of 19 subjects reacted to Ag 85, and 16 of 19 reacted to the *M. leprae* 10-kDa protein. Cells from 10 and 9 of these 19 contacts proliferated towards the *M. leprae* and BCG 65-kDa proteins, respectively. Cells from 5 of 19 lepromin-positive contacts proliferated in response to the *E. coli* 65-kDa protein, and cells from 4 of them reacted to the BCG and *M. leprae* 65-kDa proteins. Five of 19 lepromin-positive contacts reacted to the *M. leprae* 18-kDa protein.

In contrast, among the eight lepromin-negative contacts, only one reacted to Ag 85 and one reacted to the *M. leprae* and BCG 65-kDa proteins. These two subjects also showed proliferation to PPD, *M. bovis* BCG CF, and *M. bovis* BCG.

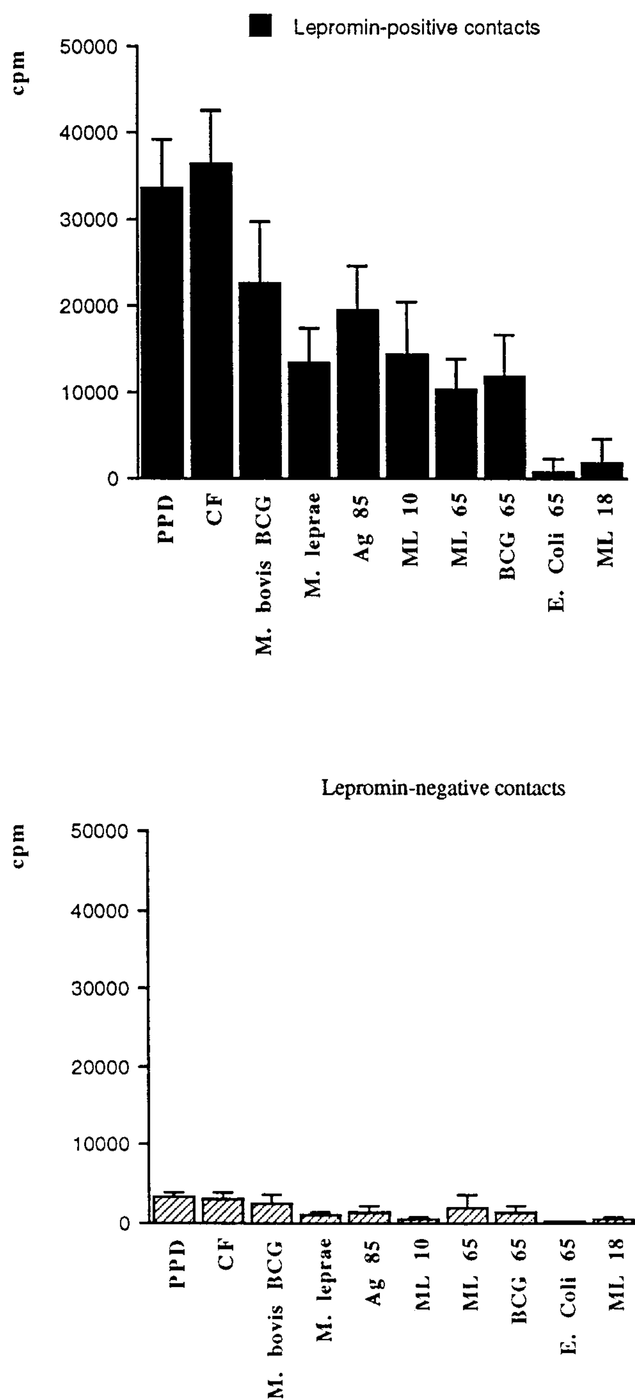
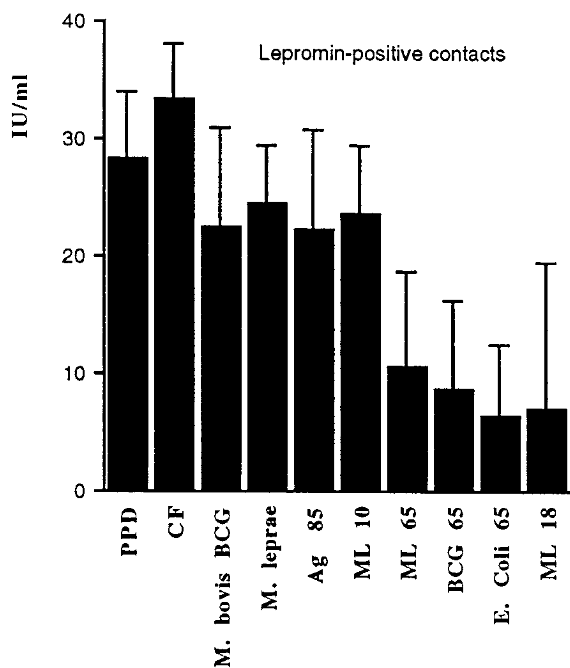
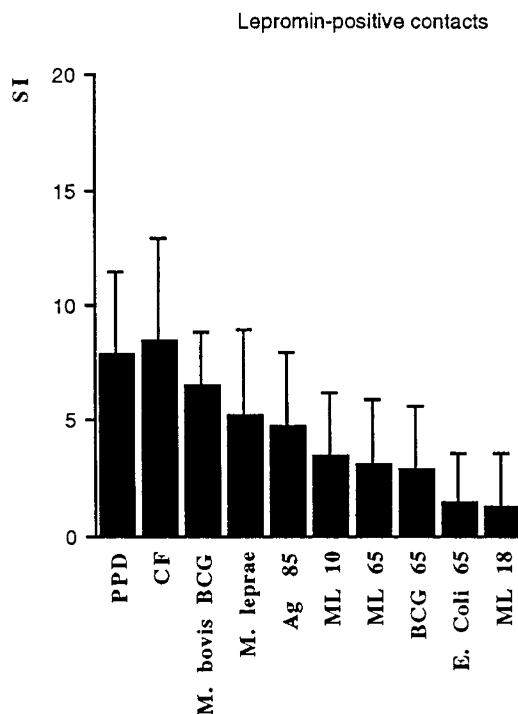


FIG. 1. Pattern of proliferative response of blood mononuclear cells recovered from leprosy contacts (mean Δcpm ± standard error of the mean). The proliferative response ([^{3}H]thymidine) was considered positive if the Δcpm was >5,000.





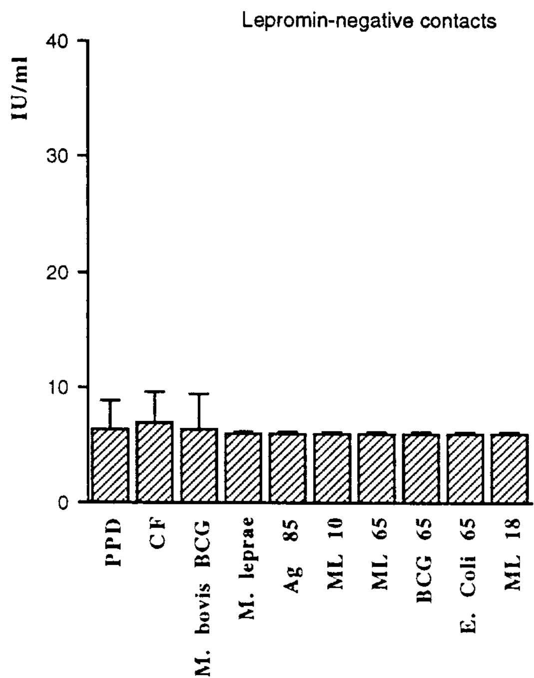


FIG. 2. Pattern of IFN-γ secretion by blood mononuclear cells from leprosy contacts (mean international units per milliliter ± standard error of the mean). IFN-γ production was considered positive if >10 IU/ml.

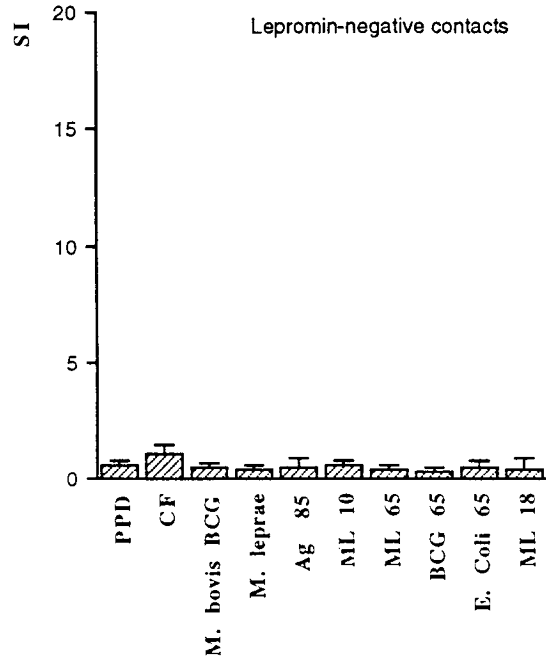


FIG. 3. Pattern of IL-2 secretion by blood mononuclear cells recovered from leprosy contacts (mean SI ± standard error of the mean). IL-2 production was considered positive if the SI was >2.

Individual IFN-γ responses to purified antigens. All lepromin-positive contacts showed a positive IFN-γ response towards PPD and BCG CF (Fig. 2), and 17 of 19 produced IFN-γ in response to *M. leprae* and BCG.

Seventeen of 19 of these contacts reacted with IFN-γ secretion to Ag 85, and 16 of 19 reacted to the *M. leprae* 10-kDa protein. Interestingly, the two lepromin-positive contacts who did not produce IFN-γ in response to *M. leprae* did not react with IFN-γ production to Ag 85 either.

Only 10 of 19 contacts produced IFN-γ in response to the

TABLE 1. Reactivity against purified antigens in leprosy contacts

Antigen	% Reactive[a]:							
	Lepromin-positive contacts				Lepromin-negative contacts			
	[^{3}H]TdR	IFN-γ	IL-2	Total[b]	[^{3}H]TdR	IFN-γ	IL-2	Total
PPD	100	100	95	100	50	12.5	12.5	50
CF	100	100	95	100	37.5	12.5	12.5	37.5
BCG	100	89.5	68.5	100	37.5	12.5	12.5	37.5
M. leprae	100	89.5	63	100	0	0	0	0
Ag 85	89.5	89.5	68.5	100	12.5	12.5	25	25
M. leprae 10-kDa protein	84	84	52.5	95	0	0	12.5	12.5
M. leprae 65-kDa protein	52	52.5	26.5	73.5	12.5	12.5	12.5	25
BCG 65-kDa protein	47.5	52.5	21	79	0	0	0	0
E. coli 65-kDa protein	26.5	26.5	10.5	47.5	0	0	0	0
M. leprae 18-kDa protein	26.5	26.5	10.5	47.5	0	0	0	0

[a] The proliferative response ([^{3}H]thymidine) ([^{3}H]TdR) was considered positive if the Δcpm was >5,000; IFN-γ production was considered positive if >10 IU/ml; IL-2 production was considered positive if the SI was >2.
[b] Positive in at least one test.

BCG and *M. leprae* 65-kDa proteins, and 5 of 19 responded to the *M. leprae* 18-kDa protein. Among the five subjects who produced IFN-γ in response to the *E. coli* 65-kDa protein, four reacted to the *M. leprae* and BCG 65-kDa proteins.

On the other hand, only one lepromin-negative contact produced a detectable IFN-γ response to PPD, BCG CF, *M. bovis* BCG, Ag 85, and the *M. leprae* 65-kDa protein.

Individual IL-2 responses to purified antigens. Figure 3 shows the pattern of IL-2 secretion by blood mononuclear cells recovered from leprosy contacts. Eighteen of 19 lepromin-positive contacts secreted IL-2 in response to PPD and BCG CF, 13 of 19 responded to *M. bovis* BCG, and 12 of 19 responded to *M. leprae*. Among the seven contacts who did not respond to *M. leprae*, four did not produce IL-2 in response to BCG either. Thirteen and 10 of 19 lepromin-positive contacts produced IL-2 in response to Ag 85 and the *M. leprae* 10-kDa protein, respectively. Only 4, 5, 2, and 2 of 19 produced IL-2 in response to the BCG 65-kDa protein, the *M. leprae* 65-kDa protein, the *E. coli* 65-kDa protein, and the *M. leprae* 18-kDa protein, respectively.

Summary of T-cell responses to purified antigens. Table 1 shows the percentages of lepromin-positive contacts who showed proliferation and/or produced TH-1 cytokines (IFN-γ or IL-2) in response to purified antigens. It shows that two antigens, i.e., Ag 85 from *M. bovis* BCG CF and the GroES-related *M. leprae* 10-kDa protein, were the most powerful antigens, eliciting a TH-1 response in 100 and 95% of lepromin-positive contacts, respectively. Fewer subjects reacted to the GroEL-related hsp 65 from *M. leprae* (73.5%) and from *M. bovis* BCG (79%). Furthermore 47.5% (9 of 19) of the subjects also reacted to GroEL from *E. coli*, and 7 of 9 reacted to both the mycobacterial and the *E. coli* products. Two lepromin-negative contacts reacted to Ag 85: one reacted with a positive response in proliferation, IFN-γ, and IL-2 secretion, and one reacted only with IL-2 secretion.

One lepromin-negative contact produced IL-2 in response to the *M. leprae* 10-kDa protein.

Precursor frequency analysis. T cells were purified from two lepromin-positive contacts. The numbers of T cells specific for Ag 85 and for the *M. leprae* 10-kDa protein are given in Table 2. Approximately one-half to one-third of the T cells of *M. leprae*-reactive cells responded either to Ag 85 or to the *M. leprae* 10-kDa protein. In contrast, only one-sixth of the T cells of *M. leprae*-specific cells responded to the BCG 65-kDa protein.

DISCUSSION

Our results show that Ag 85 from *M. bovis* BCG CF and the 10-kDa hsp from *M. leprae* are powerful inducers of a TH-1-type response in 95 to 100% of lepromin-positive contacts. Furthermore, and confirming previous findings (17, 20), precursor frequency analysis of PBMC from two lepromin-positive contacts indicates that about one-third of *M. leprae*-reactive T cells could be directed against the *M. leprae* 10-kDa protein or Ag 85.

The 10-kDa molecule from *M. leprae* is an hsp, homologous to the GroES gene product from *E. coli*, and has previously been described by Mehra et al. as a potent T-cell antigen in leprosy (20). Reactivity against the *M. leprae* 10-kDa protein parallels the response against *M. leprae* throughout the leprosy spectrum. The homologous protein in *M. tuberculosis* displays 90% identity with the *M. leprae* 10-kDa protein and is highly immunogenic in patients with tuberculous infection (3). This *M. tuberculosis* 10-kDa protein is to some extent a secreted protein because it can be found in early CF (1), and specific T-cell clones to the *M. tuberculosis* 10-kDa protein are reactive against *M. tuberculosis* CF (2). However, the 10-kDa protein sequence does not contain a classical signal peptide sequence, and it is not clear how this molecule is actually secreted. In *M. leprae* the 10-kDa hsp is expressed in cytosol and cell walls (21). Because *M. leprae* cannot be grown in vitro so far, CF of *M. leprae* is unavailable, and hence it is impossible to known whether this protein is also secreted by the leprosy bacillus.

TABLE 2. Limiting-dilution analysis of frequency of precursor T cells reactive to the *M. leprae* 10-kDa protein and Ag 85 in PBMC from two healthy lepromin-positive contacts

Antigen	Frequency of reactive cells[a]	
	Contact 1	Contact 2
M. leprae	1/5,255 (1/4,722–1/5,923)	1/5,222 (1/4,713–1/5,839)
M. leprae 10-kDa protein	1/16,174 (1/13,363–1/20,472)	1/13,522 (1/11,837–1/15,766)
Ag 85	1/8,613 (1/7,755–19,863)	1/9,835 (1/8,595–1/11,488)
BCG 65-kDa protein	1/28,865 (1/23,961–1/36,292)	ND[b]

[a] Parentheses indicate range.
[b] ND, not done.

Ag 85 is a major secreted protein antigen from *M. bovis* BCG and *M. tuberculosis* CF. It is actually a 30-32-kDa protein family with three members (85A, 85B, and 85C) which are encoded by three distinct but highly homologous genes (32). Ag 85 is also present in *M. leprae*, and the corresponding genes have been cloned and demonstrate 90% homology at the protein level with those of *M. bovis* BCG and *M. tuberculosis* (which are virtually identical) (7, 29, 30). The Ag 85 homolog from *M. bovis* BCG can be used as a alternative for T-cell analysis in leprosy. Indeed, we have demonstrated that reactivity against Ag 85 from BCG closely parallels the reactivity against whole *M. leprae* rather than that against whole *M. bovis* BCG (16) in *M. leprae*-infected subjects.

In previous work, we have reported that T cells from all lepromin-positive healthy subjects and paucibacillary leprosy patients react to Ag 85 from *M. bovis* BCG, whereas T cells from multibacillary leprosy patients do not (16), and furthermore, T cells reactive against Ag 85 are generated early during *M. leprae* infection, even before development of a positive lepromin skin test (17). In this study, cells from one lepromin-negative contact proliferated and produced IFN-γ and IL-2 in response to Ag 85. We are monitoring this person to see whether he will eventually develop a positive lepromin skin test.

The *M. leprae* 65-kDa, BCG 65-kDa, and *M. leprae* 18-kDa antigens ranked as less potent TH-1 inducers. Indeed these proteins elicited IFN-γ and IL-2 responses in only a fraction of lepromin-positive contacts (72.5, 79, and 47.5%, respectively).

TH-1 T cells also mediate delayed-type hypersensitivity reactions. The *M. leprae* and *M. tuberculosis* 10-kDa proteins have been reported to induce a strong delayed-type hypersensitivity response in sensitized guinea pigs (20, 22). Ag 85 from *M. bovis* BCG also elicits a positive skin test reaction in guinea pigs immunized with live BCG (6, 33) but with a lower magnitude than the hsp 65.

Recently we have mapped the human T-cell epitopes of Ag 85A in leprosy and tuberculosis by using 28 overlapping 20-mer synthetic peptides: regions spanning amino acids 41 to 80 and 241 to 295 demonstrated powerful and promiscuous T-cell-stimulatory properties, as measured by lymphoproliferation and IFN-γ secretion, with slight differences between healthy and diseased individuals on the one hand and differences between *M. tuberculosis*- and *M. leprae*-infected subjects on the other hand (15).

Although definitive proof has not yet been obtained in an experimental animal model, the *M. leprae* 10-kDa protein and Ag 85, inducing a TH-1 T-cell response and a positive delayed-type hypersensitivity reaction, might be valuable candidates for inclusion in a future subunit leprosy vaccine.

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