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Induction of Th1 Cytokine Responses by Mycobacterial Antigens in Leprosy

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Twelve mycobacterial antigens were compared for induction of gamma interferon (IFN-γ) secretion by human blood mononuclear cells of patients with leprosy. Fractionated *Mycobacterium leprae* antigens containing cell wall proteins or cytosolic and membrane proteins induced good IFN-γ responses in tuberculoid leprosy patients. Lipoarabinomannan from *M. tuberculosis* Erdman and *M. leprae* mycolylarabinogalactan peptidoglycan were the poorest IFN-γ inducers.

Patients with tuberculoid leprosy have positive skin-test and lymphocyte-proliferation responses to leprosy antigens, while untreated patients with lepromatous disease are anergic (26). Th1 cytokine mRNAs (21, 22, 29) have been demonstrated to be present in tuberculoid leprosy skin lesions, while levels of mRNAs for the Th2 cytokines are increased in lepromatous disease (36). It has therefore been proposed that tuberculoid leprosy involves activation of the Th1 T-cell subset and that activation of Th2 T cells in patients with lepromatous leprosy provides help for antibody production.

The signals leading to stimulation of the Th1 and Th2 subsets are still unclear (31). Some mycobacterial antigens, such as purified protein derivative (PPD), stimulate a predominantly Th1 response; other antigens, such as tetanus toxoid, induce stimulation of both Th1 and Th2 T cells (6, 8, 10). We have therefore compared mycobacterial antigens of different types, including crude, fractionated, and recombinant protein antigens from *Mycobacterium leprae*, for their ability to induce secretion of gamma interferon (IFN-γ), interleukin 4 (IL-4), and IL-5 in patients with leprosy.

Leprosy patients were recruited from the Marie Adelaide Leprosy Centre, Karachi, Pakistan ($n = 7$; all untreated), and the Hospital for Tropical Diseases, London, United Kingdom ($n = 3$; long-term treated with various drug regimens). Patients were diagnosed clinically and by evaluation of bacterial indexes in slit-skin smears; the smears of the treated patients were all negative. None of the patients showed clinical features of reactions.

The antigen panel contained *M. leprae* soluble sonicate, prepared from cobalt-irradiated armadillo-derived *M. leprae*, from the World Health Organization Immunology of Mycobacteria (IMMYC) antigen bank, *M. bovis* BCG vaccine (Glaxo strain; Evans Medeva, Liverpool, United Kingdom), and purified protein derivative (PPD) from *M. tuberculosis* (Statens Seruminstitut, Copenhagen, Denmark). Lipoarabinomannan (LAM) from *M. tuberculosis* Erdman was prepared as previously described (3). A soluble cell wall protein (PCW) fraction was obtained by spinning a sonicate of *M. leprae* at 27,000 × *g* (27); the pellet was then washed in 2% sodium dodecyl sulfate, which released the cell wall-associated proteins. A solution of soluble cytosolic and membrane proteins containing LAM and carbohydrates (SP+) was obtained as the supernatant from the spin at 27,000 × *g*; a fraction without LAM and other carbohydrates (SP−), as judged by staining with silver nitrate, was prepared by treating SP+ with DEAE-cellulose to bind the proteins. The insoluble mycolylarabinogalactan peptidoglycan (mAGP) cell wall preparation was devoid of protein and did not contain LAM (5). The recombinant *M. tuberculosis* 70-kDa, *M. leprae* 65-kDa, *M. leprae* 18-kDa, and *M. leprae* 10-kDa antigens were obtained from the World Health Organization IMMYC antigen bank. The LAM, PCW, and SP+ and SP− antigens were lipopolysaccharide free; the recombinant antigens showed various levels of contamination with endotoxin (*M. tuberculosis* 70-kDa antigen, 252 endotoxin units [EU]/mg; *M. leprae* 65-kDa antigen, 190 EU/mg; *M. leprae* 18-kDa antigen, 20 EU/mg; and *M. leprae* 10-kDa antigen, 10,000 EU/mg) as measured by enzyme-linked immunosorbent assay (ELISA; Biowhittaker, Reading, United Kingdom). Antigens were used at a final concentration of 10 μg/ml, and BCG was used at 5×10^5 bacteria per ml. The mitogen phytohemagglutinin (Difco, East Moseley, United Kingdom, or Sigma, Poole, United Kingdom) was used at 0.01 to 0.1% as a positive control.

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood, and assays were performed with 2×10^5 cells per well in 96-well plates (Nunc, Roskilde, Denmark) (7). Lymphocyte proliferation was measured by incorporation of [^{3}H]thymidine (Amersham International plc, Little Chalfont, Buckinghamshire, United Kingdom) on day 5. Positive responses were defined as the change in counts per minute (the mean counts per minute of wells with antigen minus the mean counts per minute of wells without antigen) of >2,500 cpm and a stimulation index of >3. As expected, *M. leprae* sonicate induced lymphocyte proliferation in five of six tuberculoid leprosy patients (Table 1). Positive responses were also seen in two of four lepromatous leprosy patients who had received long-term therapy with dapsone for over 30 years as well as other drugs for shorter periods. The PCW fraction and the

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TABLE 1. Lymphocyte proliferation induced by mycobacterial antigens in PBMC from leprosy patients

Antigen[a]	Lymphocyte proliferation (mean change in cpm ± SE) in patients with[b]:	
	Tuberculoid leprosy (n = 6)	Lepromatous leprosy (n = 4)
MLSON	11,455 ± 4,477	8,514 ± 4,922
BCG	8,290 ± 3,442	920 ± 379
PPD	17,162 ± 7,088	14,871 ± 4,324
PCW	10,025 ± 4,590	10,761 ± 6,087
SP−	7,254 ± 4,828	14,878 ± 9,710
SP+	7,987 ± 2,558	4,738 ± 1,554
LAM	2,262 ± 1,213	466 ± 441
mAGP	1,679 ± 924	539 ± 377
70kDa	3,025 ± 2,100	2,313 ± 1,838
65kDa	3,622 ± 2,103	3,102 ± 2,448
18kDa	12,209 ± 6,104	6,904 ± 4,322
10kDa	3,779 ± 2,442	685 ± 402

[a] MLSON, *M. leprae* soluble sonicate; BCG, *M. bovis* BCG vaccine (Glaxo); PPD, PPD of *M. tuberculosis*; LAM, LAM from *M. tuberculosis* Erdman; 70kDa, *M. tuberculosis* recombinant 70-kDa antigen; 65kDa, *M. leprae* recombinant 65-kDa antigen; 18kDa, *M. leprae* recombinant 18-kDa antigen; 10kDa, *M. leprae* recombinant 10-kDa antigen.

[b] The change in counts per minute was defined as the mean counts per minute in wells with antigen minus the mean counts per minute in wells without antigen. The levels of background proliferation in the absence of antigen were not significantly different in the two groups (mean ± standard error, 1,592 ± 306 cpm for the tuberculoid group and 904 ± 345 cpm for the lepromatous group; by Student's t test, $P = 0.18$).

cytosolic protein fractions (SP+ and SP−) induced proliferation in the majority of the *M. leprae* responders. Minimal proliferative responses to LAM or to the insoluble mAGP were observed. The recombinant antigens induced variable proliferation, the highest responses being seen with the *M. leprae* 18-kDa antigen. Thus, all the antigens could induce a proliferative response in at least a portion of the leprosy patients.

To measure IFN-γ, supernatants were collected on day 5 and frozen until tested. IFN-γ was measured by ELISA (13) with a human IFN-γ international standard (National Institute for Biological Standards and Control, South Mimms, United Kingdom) to calibrate the assay, which detects as little as 3 IU of IFN-γ per ml. PBMC supernatants from cultures incubated without antigen contained <5 IU of IFN-γ per ml. IFN-γ was present at high concentrations in all the cultures stimulated with phytohemagglutinin. The responses to *M. leprae* sonicate, BCG, and PPD were more variable (Fig. 1A and B).

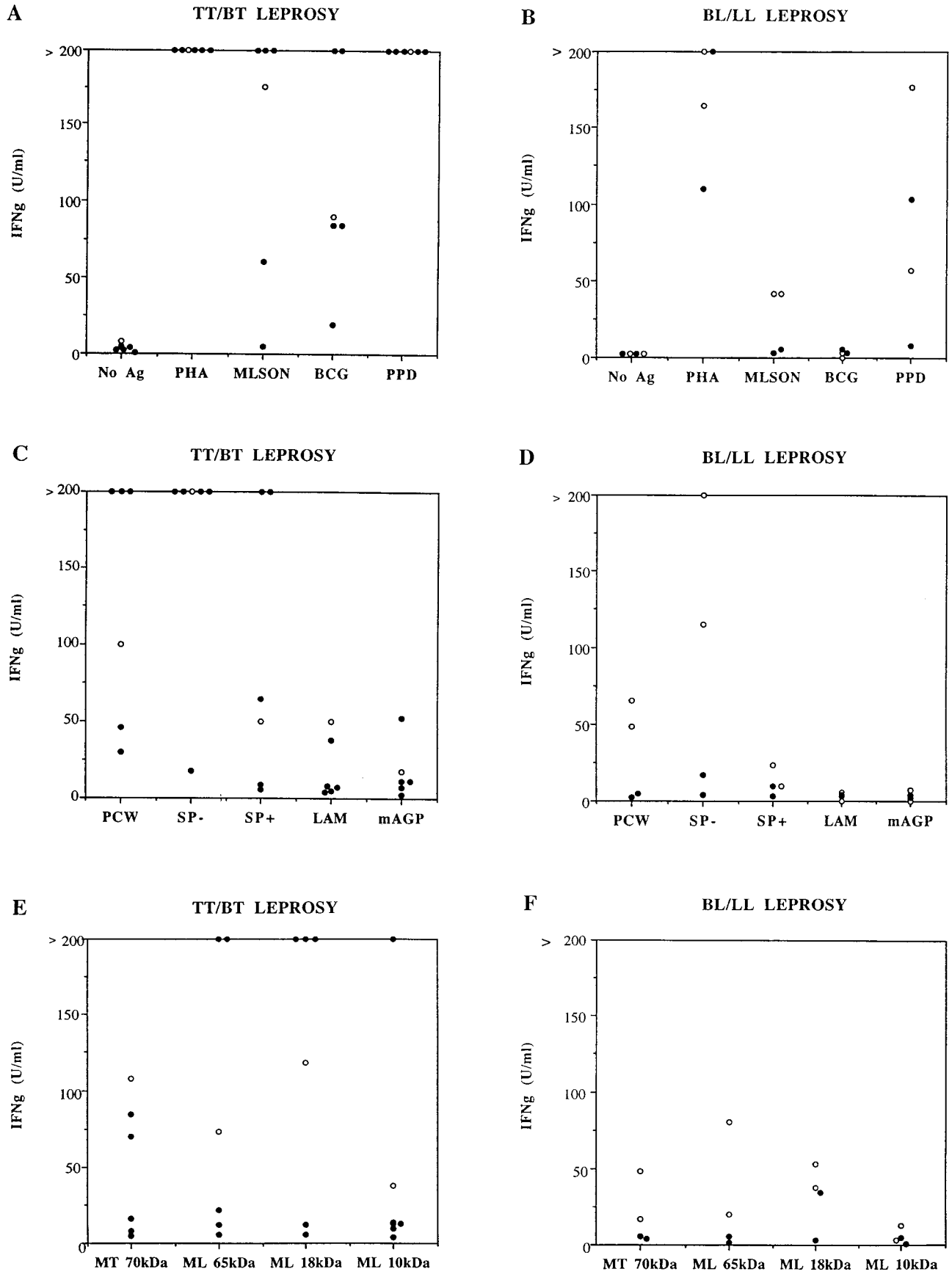
The fractionated leprosy antigens showed differing abilities to induce IFN-γ secretion. Overall, the strongest IFN-γ responses were observed with SP−, although four tuberculoid leprosy patients responded well to SP+, and the soluble PCW fraction also induced good responses. LAM and the insoluble cell wall peptidoglycan mAGP, which had failed to induce good proliferative responses, induced low IFN-γ responses, particularly in the lepromatous leprosy patients (Fig. 1C and D). All the antigenic preparations which had induced lymphocyte transformation were therefore able to induce secretion of IFN-γ.

With the individual recombinant antigens, the strongest responses were observed in the tuberculoid leprosy patients (Fig. 1E and F); weaker responses were observed in lepromatous patients, even though the 70-kDa antigen used was from *M. tuberculosis*, and the 65- and 10-kDa antigens are highly cross-reactive with the molecules of the same molecular masses in *M. tuberculosis* and *M. bovis* BCG (18, 32). Thus, all four recombinant antigens were capable of inducing a Th1 T-cell response.

We also tested for IL-4 and IL-5 in some 48-h supernatants by ELISA using commercial reagents (R & D Systems, Abingdon, United Kingdom, and Pharmingen, Cambridge Biosciences, Cambridge, United Kingdom). Phytohemagglutinin supernatants contained both IL-4 (mean amount ± standard error, 98 ± 33 pg/ml; n = 6) and IL-5 (831 ± 198 pg/ml; n = 9). PBMC from three untreated leprosy patients produced low levels of IL-4 (<10 pg/ml) in response to some fractionated or recombinant antigens. Minimal IL-5 secretion (<58 pg/ml) was induced in six of nine patients by PPD, SP+, and the 65-kDa or 10-kDa antigen (results not shown). It might therefore be necessary to use a more sensitive assay, such as the ELISPOT assay, to demonstrate secretion of these cytokines by individual T cells (8) or to detect mRNA by reverse transcription-PCR. In a recent study, Misra et al. (19) were able to demonstrate Th2 and ThO cytokine patterns in PBMC from lepromatous leprosy patients. However, although lepromatous leprosy involves extensive antibody production (2, 12), immunoglobulin G (IgG) antibodies to the *M. leprae* 18-kDa antigen are of the IgG1 and IgG3 subclasses rather than of the IgG4 subclass, as might be expected in a Th2 response (14). Lepromatous leprosy patients may therefore have depressed Th1 responses without activation of Th2 T cells in the peripheral blood, an observation similar to the recent observations of depressed Th1 responses without enhanced Th2 responses in patients with tuberculosis (37).

This study has provided further evidence that tuberculoid leprosy involves an IFN-γ response to a broad range of antigens which may be produced not only by αβ T-cell receptor $CD4^+$ T cells but also by $CD8^+$ and γδ T cells (30). Secretion of IFN-γ induced by the 65-kDa, 32-kDa, 18-kDa, and 10-kDa antigens has been described previously (16). A Th1 T-cell response may be protective; mice protected by vaccination with *M. leprae* make IFN-γ mRNA (34), and injection of IFN-γ into leprosy skin lesions reduces the bacterial load (15, 23). The only antigens which failed to induce good Th1 responses were the cell wall mAGP, a complex carbohydrate structure linked to peptidoglycan (5), and the carbohydrate LAM. LAM from *M. tuberculosis* H37Ra failed to induce mRNA for IFN-γ in PBMC from tuberculin reactors or patients with tuberculosis (1). LAM, presented by CD1 molecules, can be recognized by αβ $CD4^-$ $CD8^-$ T cells (33), although the antigen-presenting cells in the PBMC cultures may not have expressed CD1. The removal of LAM and other carbohydrates from the cytosolic fraction appeared to enhance the T-cell response, perhaps because of the immunosuppressive properties of LAM (20), although contaminating endotoxin in the recombinant antigens

FIG. 1. IFN-γ (IFNg) secretion induced by mycobacterial antigens in patients with leprosy. PBMC from tuberculoid (TT) or borderline tuberculoid (BT) leprosy patients (A, C, and E) and from borderline lepromatous (BL) or lepromatous (LL) leprosy patients (B, D, and F) were incubated with or without antigens, and supernatants collected at 5 days were assayed for IFN-γ by ELISA. No Ag, PBMC incubated without antigen (negative control); PHA, phytohemagglutinin (positive control); MLSON, *M. leprae* soluble sonicate; BCG, *M. bovis* BCG vaccine (Glaxo); PPD, PPD of *M. tuberculosis*; LAM, LAM from *M. tuberculosis* Erdman; MT 70kDa, *M. tuberculosis* recombinant 70-kDa antigen; ML 65kDa, *M. leprae* recombinant 65-kDa antigen; ML 18kDa, *M. leprae* recombinant 18-kDa antigen; ML 10kDa, *M. leprae* recombinant 10-kDa antigen. Filled circles represent untreated leprosy patients, and open circles represent patients who had received long-term therapy.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did not appear to block either lymphocyte proliferation or IFN-γ secretion.

Although the number of lepromatous patients studied was small, patients who had received long-term therapy gave better IFN-γ responses than untreated patients. Recovery of skin-test positivity and lymphocyte proliferation have been observed in lepromatous leprosy patients following therapy (4, 9, 35). A sequential study of cytokine production before, during, and after therapy would be needed to confirm this recovery of Th1 responsiveness.

The strong proliferative responses and IFN-γ secretion induced by the cell wall or cytosol and membrane antigens imply that these preparations include important antigens recognized by T cells. The cell wall may include proteins of 7, 16, and 28 kDa (18), as well as the histoprotein H2b (25). The cytosol fraction contains both the *M. leprae* 10-kDa antigen, the major cytosol protein (27), described as an immunodominant antigen in tuberculoid leprosy (17), and the 18-kDa antigen, which is strongly recognized by healthy individuals who have been in contact with leprosy patients (7). Other secreted antigens, such as the 30- to 32-kDa Ag85 proteins (24), may be present in the cytosolic preparation. A recent study of lepromin-positive individuals who have been in contact with leprosy patients showed that the *M. leprae* 10-kDa antigen and Ag85 were the predominant antigens inducing a Th1 response (16). Studies using the mouse footpad model have shown that immunization with cell wall proteins can induce immunity against *M. leprae* (11, 28). Thus, the cell wall and cytosolic proteins may be useful antigens to evaluate as skin-test reagents in humans. As these antigens can induce Th1 T-cell responses, they may also play a role in the protective immune response against leprosy.

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