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Leprosy patients with lepromatous disease recognize cross-reactive T cell epitopes in the *Mycobacterium leprae* 10-kD antigen

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SUMMARY

T cell responses play a critical role in determining protective responses to leprosy. Patients with self-limiting tuberculoid leprosy show high T cell reactivity, while patients with disseminated lepromatous form of the disease show absent to low levels of T cell reactivity. Since the T cell reactivity of lepromatous patients to purified protein derivative (PPD), a highly cross-reactive antigen, is similar to that of tuberculoid patients, we queried if lepromatous patients could recognize cross-reactive epitopes in *Mycobacterium leprae* antigens as well. T cell responses were analysed to a recombinant antigen 10-kD (a heat shock cognate protein) which is available from both *M. tuberculosis* (MT) and *M. leprae* (ML) and displays 90% identity in its amino acid sequence. Lymphoproliferative responses were assessed to ML and MT 10 kD in newly diagnosed leprosy patients (lepromatous, $n = 23$; tuberculoid, $n = 65$). Lepromatous patients showed similar, but low, lymphoproliferative responses to ML and MT 10 kD, while tuberculoid patients showed much higher responses to ML 10 kD. This suggests that the tuberculoid patients may be recognizing both species-specific and cross-reactive epitopes in ML 10 kD, while lepromatous patients may be recognizing only cross-reactive epitopes. This was further supported by linear regression analysis. Lepromatous patients showed a high concordance in T cell responses between ML and MT 10 kD ($r = 0{\cdot}658$; $P < 0{\cdot}0006$) not observed in tuberculoid patients ($r = 0{\cdot}203$; $P > 0{\cdot}1$). Identification of cross-reactive T cell epitopes in *M. leprae* which could induce protective responses should prove valuable in designing second generation peptide-based vaccines.

Keywords leprosy 10-kD recombinant antigen T cell epitopes cross-reactive

INTRODUCTION

Mycobacterium leprae remains one of the few bacterial pathogens of humans that has not been cultivated *in vitro* and therefore development of a successful vaccine depends on the identification of antigens and epitopes that induce protective responses across the leprosy disease spectrum. Several biochemical, immunological and molecular approaches have been recently used for identification and characterization of protein antigens of the leprosy bacillus [1–4]. It has long been recognized that leprosy patients with self-limiting tuberculoid leprosy show high T cell reactivity, while patients with disseminated lepromatous form of the disease show absent to low levels of T cell reactivity [5]. T cell hyporesponsiveness in patients with lepromatous disease is highly antigen-specific, since this group of patients show normal responses to T cell mitogens and unrelated recall antigens [6]. Among the 10 or more dominant *M. leprae* T cell antigens which have been characterized and cloned [4], heat shock proteins (hsp) have been shown to induce strong T cell responses in leprosy patients with tuberculoid or self-limiting disease [7]. Among the hsp, *M. leprae* 10 kD has been shown to induce strong T cell responses across the leprosy spectrum [8,9] as well as in leprosy contacts [10]. We therefore queried if the lepromatous patients were recognizing cross-reactive rather than *M. leprae* species-specific epitopes in *M. leprae* 10 kD.

Mycobacterium leprae 10 kD is a homologue of the GroES gene product of *Escherichia coli* and the human chaperonin 10 [11] and participates in protein folding [12]. The structure of the *M. leprae* 10 kD has already been elucidated [13] and the amino acid composition and sequence determined [13]. A highly homologous hsp with 90% amino acid homology to *M. leprae* 10 kD has been characterized in *M. tuberculosis* [14]. Both *M. leprae* and *M. tuberculosis* 10 kD preparations are available as recombinant antigens from the WHO/TDR antigen bank. We compared the two 10-kD recombinant antigens in parallel across the leprosy spectrum and in controls to see if lepromatous patients were recognizing

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cross-reactive determinants at the T cell level in the two 10-kD preparations.

PATIENTS AND METHODS

Patients and controls

Newly diagnosed leprosy patients presenting at the Marie Adelaide Leprosy Centre (MALC) were recruited to our studies and have been described in detail elsewhere [15]. Patients are diagnosed clinically as well as histologically on a 4-mm punch biopsy taken from the edge of an active lesion [15]. Eighty-eight patients from across the leprosy spectrum (lepromatous and borderline lepromatous (L) = 23; borderline tuberculoid and tuberculoid (T) = 65) who had not been treated for leprosy previously were included in the study. Healthy endemic controls (EC = 19) who were employees of Aga Khan University (AKU) and had no previous history of exposure to leprosy were used as the control group. Ethical approval was obtained from both AKU and MALC Human Rights Protection Committee. Written/oral consent as appropriate was obtained from both patients and control groups.

Antigens

Mycobacterium leprae 10 kD (ML10K; batch ML10-2) and *M. tuberculosis* 10 kD (MT10K; batch MT10-2) antigens were obtained from the WHO reference reagent bank through the courtesy of Dr Jan van Embden. The *M. leprae* 10 kD (ML10K) contains 603 U/mg of endotoxin and *M. tuberculosis* 10 kD (MT10K) contains 698 U/mg of endotoxin. Purified protein derivative (PPD; lot R44) was obtained from Statens Seruminstitut (Copenhagen, Denmark) and *M. leprae* sonicate (ML) lot CD197 was obtained through the courtesy of Dr J. Colston (NIMR, UK).

Antisera

Five millilitres of blood collected from leprosy patients were allowed to separate overnight at 4°C. Serum was removed and centrifuged at 400 ***g*** for 15 min; the clear supernate was distributed in small aliquots and frozen at −70°C before use.

Assay of lymphocyte blastogenesis

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood (30 ml) by density sedimentation over Ficoll–Hypaque. Cells were washed three times with medium (RPMI 1640; BioWhittaker, Walkersville, MD). Cells were counted and suspended in complete medium (RPMI 1640 with 2 mM L-glutamine, 100 mg/ml gentamycin, 15 mM HEPES and 20% autologous human plasma). Cells (2×10^4/well) were placed in round-bottomed microtitre tissue culture plates (Flow Labs, Irvine, UK). PPD (10 μg/ml), *M. leprae* sonicate (ML; 10 μg/ml), *M. leprae* 10 kD (ML10K; 5 μg/ml) and *M. tuberculosis* 10 kD (MT10K; 5 μg/ml) were added to triplicate wells for each donor. Control wells received medium alone. The cultures were incubated for 5 days in 5% CO_2 at 37°C. ^{3}H-thymidine (1 μC; specific activity 6·7 C/mmol (Amersham, Aylesbury, UK)) was added to each culture well for the final 24 h. Cells were harvested after 18 h with a PHD harvester (Cambridge Technology, Cambridge, MA) and ^{3}H-thymidine incorporation was measured in a scintillation counter. Results were expressed as mean ct/min of the triplicates. A response was considered positive if the counts (Δcpm) incorporated in experiments were ≥ 2000 ct/min higher than counts incorporated in cells cultured with medium alone. Spontaneous incorporation of ^{3}H-thymidine in cultured cells ranged between 500 and 1000 ct/min.

Assay for interferon-gamma in culture supernatants of stimulated PBMC

Supernatants were collected from stimulated cells after 5 days and assayed for interferon-gamma (IFN-γ) secretion by an ELISA-based assay. All reagents for the IFN-γ assay were obtained from Pharmingen (San Diego CA). Plates were coated with 100-μl volumes containing 2·5 μg/ml of mouse monoclonal anti-human IFN-γ (capture antibody) in carbonate buffer 0·1 M pH 8·2 and incubated overnight at 4°C. The plates were blocked with 3% bovine serum albumin (BSA) to quench the remaining binding sites. Plates were subsequently incubated with the reference and test samples and further incubated for 2 h at 37°C. The plates were then incubated with detecting antibody (biotinylated mouse anti-human IFN-γ). The revealing probe was avidin conjugated to horseradish peroxidase (HRP; Sigma, St Louis, MO). Plates were washed with PBS containing Tween-20 (0·05%) between each incubation. OPD (Sigma) was used as a substrate for colour development. The reaction was stopped with 4 M H_2SO_4 and the reaction read at 410 nm in a Titertek plate reader MR 5000 (Dynatech, VA).

Statistical analysis

Statistical analysis was done on an Apple Macintosh microcomputer using Statview software packages.

RESULTS

Immune characteristics of the study groups

A total of 88 newly diagnosed leprosy patients was tested for lymphocyte proliferation, of which 23 were classified as multibacillary lepromatous leprosy (L) and 65 as paucibacillary tuberculoid (T) leprosy. In addition, 19 healthy EC were also studied. Lymphocyte proliferation was assessed by incorporation of ^{3}H-thymidine and a positive response defined as > 2000 Δct/min. Figure 1 shows both the magnitude (Fig. 1a) as well as the frequency of positive response (Fig. 1b) to ML and PPD in the three study groups. Leprosy patients showed the expected antigen-specific response profile for both the intensity and percentage positivity of responses. The magnitude of response to ML was markedly lower in patients with lepromatous disease compared with patients with tuberculoid disease. As expected, both groups showed strong positive responses to PPD. This characteristic immune profile of our study group was therefore reassuring for further analysis with the recombinant antigens. Bacille Calmette–Guérin (BCG)-vaccinated healthy EC ($n = 19$) showed PPD and ML responses comparable to leprosy patients with tuberculoid disease.

Comparison of T cell responses across leprosy spectrum to ML and MT10K recombinant antigen

Figure 2 shows the magnitude (Fig. 2a) and the frequency of responses (Fig. 2b) to the two cross-reactive ML10K and MT10K recombinant antigens. As expected, there was significantly higher intensity of lymphoproliferative responses to ML10K in tuberculoid leprosy patients (*t*-test; $P < 0·0002$) compared with patients with lepromatous disease (Fig. 2a). When the magnitude of proliferative responses to ML10K were compared with MT10K antigens within the same groups, it was interesting to note that while lepromatous patients showed the same magnitude of T cell responses to both ML and MT 10-kD antigens, leprosy patients with tuberculoid disease showed much higher responses to ML10K

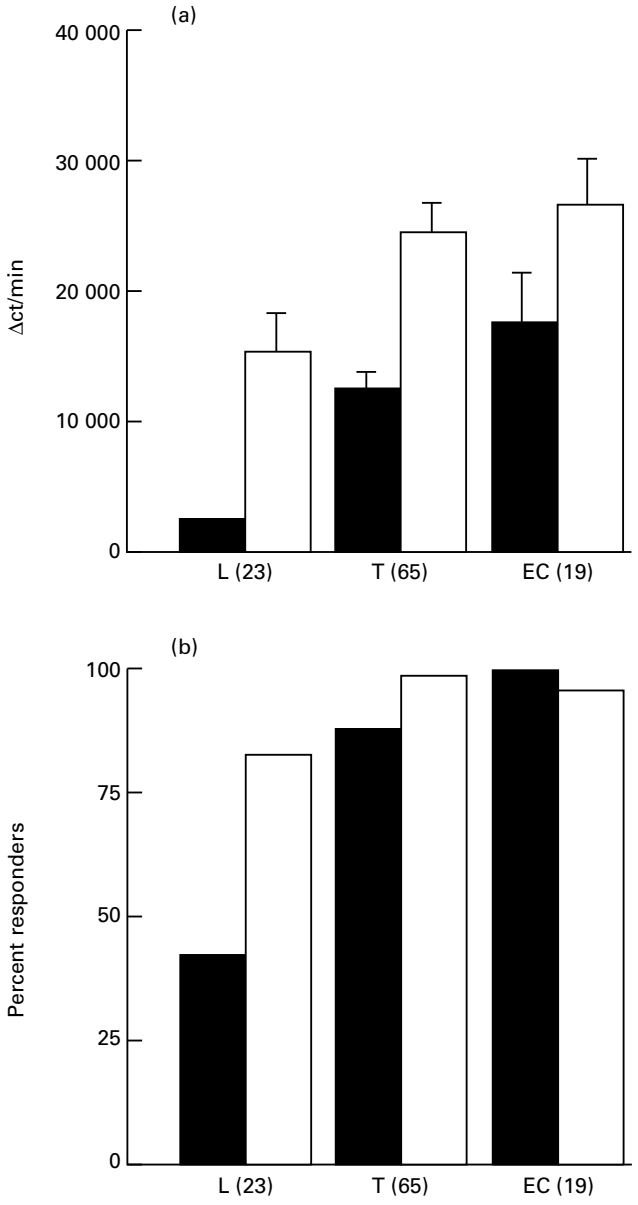

Fig. 1. Immune profile of leprosy patients and healthy endemic controls (EC). Lymphocyte proliferative responses to *Mycobacterium leprae* sonicate (■; 10 μg/ml) and purified protein derivative (PPD; □; 10 μg/ml) in lepromatous and borderline lepromatous leprosy patients (L = 23), borderline tuberculoid and tuberculoid leprosy patients (T = 65) and healthy EC (EC = 19). (a) Lymphoproliferative responses as mean Δct/min of the groups ± 1 s.e.m. (b) Frequency of positive responses using > 2000 Δct/min as a cut off.

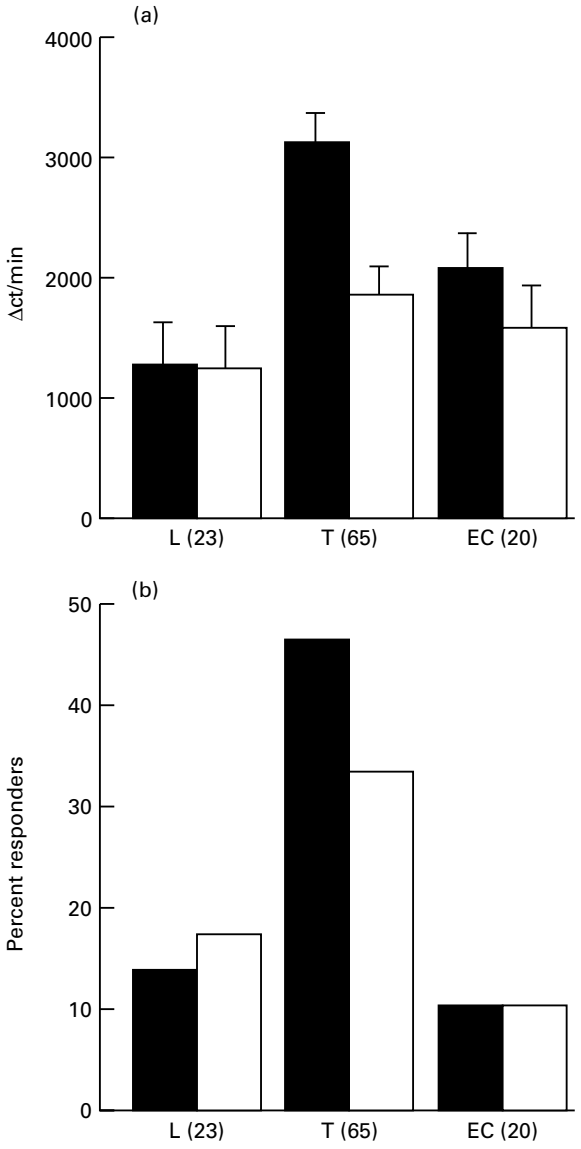

Fig. 2. Lymphoproliferative responses to *Mycobacterium leprae* (■; 5 μg/ml) and *M. tuberculosis* 10 kD (□; 5 μg/ml) recombinant antigens. All other parameters are as described in Fig. 1.

than to MT10K. These results suggest that responses to ML10K are part of the specific T cell response to *M. leprae*, in that they are down-regulated in lepromatous patients compared with patients with tuberculoid disease, while responses to MT10K may be related to recognition of cross-reactive T cell epitopes.

The frequency of positive responses to both r-10K antigens (Fig. 2b) paralleled the magnitude of response. Tuberculoid patients again showed stronger recognition of ML10K (46·1%) compared with MT10K (33·8%), while lepromatous patients showed a marginally higher frequency of response to MT10K. Although none of these differences were statistically different (χ^2 analysis), they do support the conclusion that T cell responses to ML10K in tuberculoid leprosy patients are part of the antigen-specific response to *M. leprae* antigens, while patients with lepromatous disease may be recognizing a restricted set of cross-reactive epitopes.

The EC group showed positive responses to both ML and MT10K antigens, and while the intensity of response (Fig. 2a) was slightly higher to ML10K compared with MT10K, the percentage responders was similar. When a group of 11 non-BCG-vaccinated donors was tested in London they also showed a high frequency of positive responses (54·4%) to ML10K, with no apparent correlation with responses to *M. leprae* sonicate or PPD (data not shown). These results suggest that the responses in the control group may be unrelated to *M. leprae* exposure.

Lepromatous patients recognize cross-reactive epitopes in ML10K

To address the issue of recognition of cross-reactive epitopes, we carried out correlation analysis of the lymphocyte proliferative response to the two antigens in leprosy patients (Fig. 3). Although lepromatous patients showed a low frequency of positive lymphoproliferative responses to the 10K antigens (Fig. 3a), a strong correlation was observed between responses to the ML and the MT10K antigens ($r = 0{\cdot}658$; $P = 0{\cdot}0006$). This correlation was not observed in the leprosy patients with tuberculoid disease ($r = 0{\cdot}203$; $P > 0{\cdot}1$).

These results suggest that leprosy patients with lepromatous disease were probably responding to common epitopes in ML10K, while the patients with tuberculoid disease were responding to both cross-reactive as well as species-specific epitopes in ML10K.

Lepromatous patients release IFN-γ in response to cross-reactive epitopes in ML10K

In order to investigate if lymphocyte proliferation in response to the 10K recombinant antigens in patients with lepromatous disease was related to a Th1 response, we assessed the concentration of IFN-γ in the supernatants of stimulated PBMC. Figure 4 shows the lymphoproliferative (Fig. 4a) and IFN-γ responses (Fig. 4b) to ML and the recombinant 10K antigens. Interestingly, *M. leprae* sonicate, which resulted in the highest proliferative responses, resulted in the lowest mean concentration of IFN-γ in supernatants of stimulated cells from patients with lepromatous disease. Since *M. leprae* sonicate is a mixture of several antigens, these results may be reflecting differential ability of different antigens to induce IFN-γ secretion. More importantly, our results clearly demonstrate that patients with lepromatous disease are able to release IFN-γ in response to the cross-reactive epitopes in ML10K as well as MT10K. It is also striking that the mean IFN-γ release in response to ML10K was equivalent or higher than that observed with the whole *M. leprae* sonicate, indicating that ML10K may be inducing the strongest Th1 response in patients with lepromatous disease. This is further illustrated in Table 1, where results are shown for a representative panel of seven donors with lepromatous diseases. All donors showed equivalent or higher responses to ML10K compared with ML (*M. leprae* whole sonicate). Epitope mapping is in progress to elucidate the cross-reactive peptides responsible for induction of Th1 response in patients with lepromatous disease.

Table 1. IFN-γ secretion to the ML10K and *Mycobacterium leprae* sonicate

Patient ID*	ML10K ΔIFN-γ† (pg/ml)	ML‡ ΔIFN-γ† (pg/ml)
LS1081	23	2·9
LS1231	2·3	0·18
LS1234	55	15
LS1241	0	2·9
LS1277	12·2	2·8
LS1289	1·0	2·3
LS1290	79	11·6

* All patients had the lepromatous form of the disease.

† IFN-γ was determined in supernatants collected 5 days after antigen stimulation. A cut off of 10 pg/ml after deducting spontaneous secretion was considered positive. Positive results are underlined.

‡ ML, *Mycobacterium leprae* sonicate.

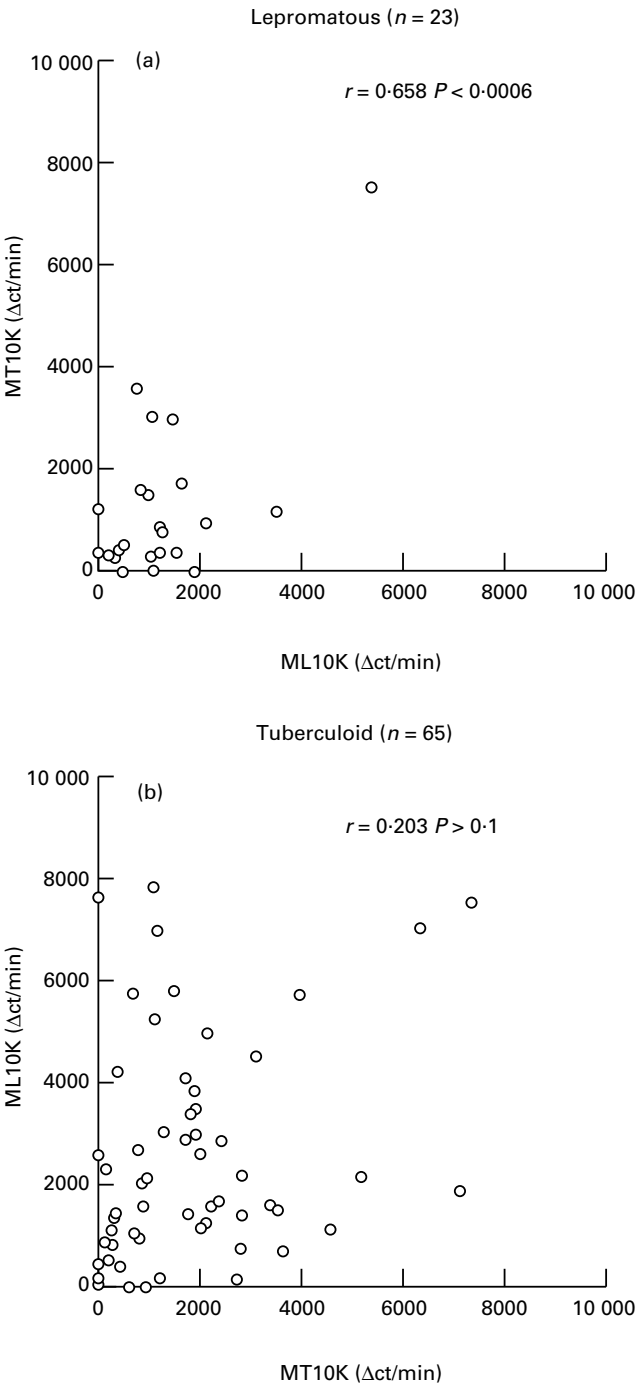

Fig. 3. Correlation of *Mycobacterium leprae* and *M. tuberculosis* 10-kD recombinant antigens in lepromatous (a) and tuberculoid (b) leprosy patients. Linear regression analysis was carried out to determine the relationship between lymphoproliferative responses to ML and MT 10-kD antigens. Results are expressed as Δct/min. The regression (r), and P values are given for each patient group.

DISCUSSION

For development of a successful vaccine it would be important to identify and incorporate cross-reactive antigens or epitopes that are recognized across the leprosy spectrum. The most important

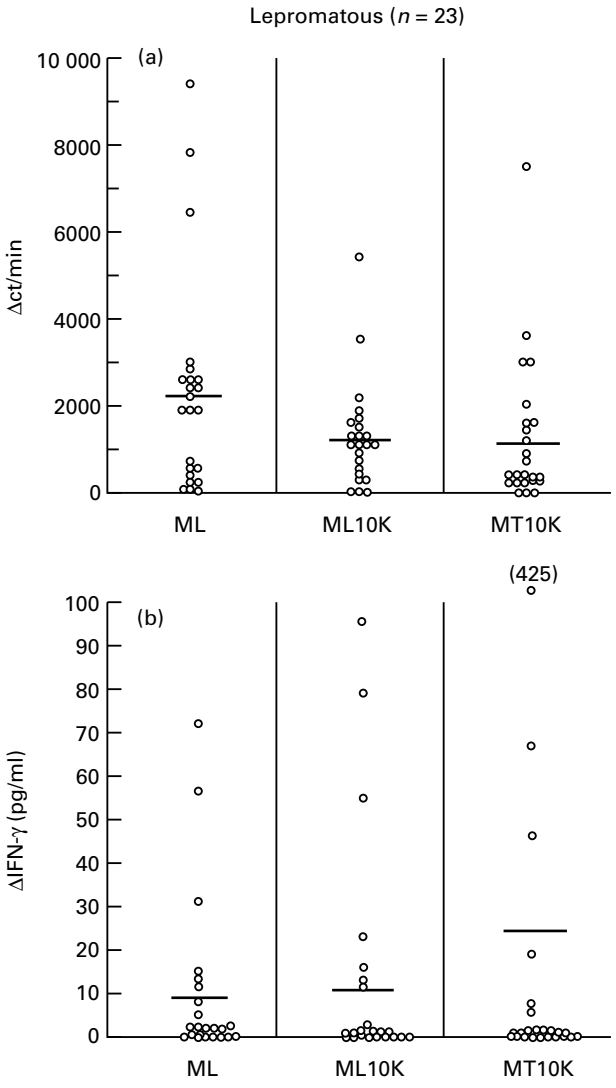

Fig. 4. Secretion of IFN-γ in response to *Mycobacterium leprae* sonicate (ML), *M. leprae* and *M. tuberculosis* (MT) 10-kD recombinant antigens by peripheral blood mononuclear cells (PBMC) from patients with lepromatous disease. Supernatants were collected after 5 days of stimulation with the ML and MT 10 kD (5 μg/ml) and assayed for IFN-γ release by T cells. Results are shown as a scattergram, each circle represents one donor. The horizontal line indicates the mean for each group. (b) The number in parentheses indicates the value for that particular donor.

contribution of our studies was the demonstration that lepromatous patients can develop protective T cell responses to certain cross-reactive epitopes present in *M. leprae* 10K, as evidenced by lymphoproliferative responses and IFN-γ secretion, which are indicators of Th1 responses associated with protection against intracellular pathogens.

Both ML and MT10K antigens have been reported as dominant T cell antigens in leprosy and tuberculosis, respectively [8,16]. Recombinant ML10K has been reported to be a dominant T cell antigen in patients with tuberculoid disease and almost 1/3 of the T cells in a lepromin-positive patient contact were shown to be ML10K-specific [8]. However, only negligible reactivity was reported in patients with lepromatous disease [8]. We report much higher frequencies of positive responses to ML10K in lepromatous patients. The earlier studies were conducted with native ML10K. Without directly comparing the immune responses to the native and recombinant forms of the antigens it is not possible to say whether these differences are related to the presence of additional contaminants in the recombinant preparation of the ML 10-kD antigen. The recombinant preparations of ML and MT10K that we have used in this study had considerable but equivalent amounts of endotoxin. However, when we compared these preparations with an endotoxin-free preparation of the same antigens (gift of Dr P. Brennan, Denver, CO), there was no difference in the lymphocyte proliferation or IFN-γ release, indicating that endotoxin did not have an effect on at least these two T cell functions (data not shown). All the leprosy patients included in our study were newly diagnosed and therefore chemotherapy could not have contributed to immunological recovery in lepromatous patients. The other more likely explanation may be that we used a much larger panel ($n = 23$) of patients compared with the previous study where a small group ($n = 11$) was analysed, which may have biased the results since the frequency of response is relatively low (13%) in this group of patients.

Recognition of ML and MT10K was highly concordant in patients with lepromatous disease, but this was not the case with leprosy patients with tuberculoid disease. This suggests that lepromatous patients were recognizing common epitopes in the 10K preparations. Although tuberculoid patients showed a much higher frequency of response to both ML10K and MT10K, there was no correlation in responses to the two 10K antigens, suggesting that this group of patients was recognizing both common and species-specific epitopes. An earlier study had also suggested that tuberculoid patients recognize both species-specific and cross-reactive epitopes using peptides containing either common or specific determinants in the NH2 terminal end [8]. A second report has shown that the NH2 terminal end is the most immunodominant region in patients with tuberculoid disease and demonstrates a DRB5*01 restriction for recognition of these epitopes [9]. However, recognition by patients with lepromatous disease has not been addressed previously, probably due to the low frequency of positive responses in these patients.

The most significant observation in our studies was that cross-reactive epitopes were able to induce a Th1 response. Recognition of both ML10K and MT10K by patients with lepromatous disease resulted in release of IFN-γ. What was surprising is that the magnitude of IFN-γ response was equivalent to or higher than that observed with the whole *M. leprae* sonicate. This would suggest that either 10K is a dominant Th1 activator in *M. leprae*, or that there is a balance of stimulatory and suppressive activities in the antigenic mixture of *M. leprae* sonicate. Results in Table 1 suggest that this may indeed be the case, since ML10K results in much higher IFN-γ response compared with ML in the same donors. Epitope mapping of the determinants in ML10K and MT10K recognized by patients with lepromatous patients would be important in understanding the nature of T cell reactivity in this group of patients. We are currently identifying the epitopes recognized by T cells in patients with lepromatous disease using 15mer peptides from these recombinant antigens. These epitopes may provide useful candidates for second-generation peptide-based vaccines for leprosy.

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