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Human T cell responses to peptides of the *Mycobacterium leprae* 45-kD serine-rich antigen

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SUMMARY

In order to identify T cell epitopes within the *Mycobacterium leprae* 45-kD serine-rich antigen, we analysed responses to overlapping 17-mer peptides encompassing the whole antigen in non-exposed UK controls, Pakistani leprosy patients and tuberculosis patients in both the United Kingdom and Pakistan. This antigen has been described as *M. leprae*-specific, although it has a hypothetical homologue in *M. tuberculosis*. Human peripheral blood mononuclear cells were stimulated with peptide for 5 days and IFN-γmeasured in supernatants by ELISA. Some peptides were recognized more frequently by T cells from tuberculoid leprosy patients than those from UK controls, suggesting that such T cell epitopes might have diagnostic potential, while other peptides induced greater responses among UK control subjects. Short-term cell lines confirmed that these assays detected specific T cell recognition of these peptides. However, many tuberculosis patients also recognized these potentially specific peptides suggesting that there could be a true homologue present in *M. tuberculosis*.

Keywords cell mediated immunity (CMI) diagnosis interferon-gamma (IFN-γ) *M. leprae* peptides

INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. Leprosy patients present with a spectrum of clinical disease that depends on the ability of the host to make a cellular immune response to the bacterium [1]. At one pole of the spectrum is tuberculoid leprosy (TT), which is associated with strong cell mediated immunity (CMI) and delayed type hypersensitivity (DTH) responses to *M. leprae.* In contrast, at the other end of the disease spectrum is lepromatous leprosy (LL) where the disease is disseminated throughout the body.

Leprosy is still considered a public health problem in 32 countries; however, 16 of these from the African, Asian and South American continents are responsible for 92% of all registered patients [2]. Leprosy cases are distributed globally unevenly. The application of multi-drug therapy (MDT) has changed the picture of leprosy, with the numbers of registered cases worldwide falling from 1·3 million in 1995 to 641 091 in 2000 [3,4]. Despite these statistics leprosy still poses some challenges. The biggest challenge for achieving elimination is the fact that even though the prevalence of leprosy is decreasing the same is not true for incidence, which has remained the same [5]. In addition, because of the long incubation period before the disease appears, many more new leprosy patients will continue to emerge in endemic countries for many years.

What makes the control of leprosy difficult is that no specific and reliable immunological tool is available to be used for detection of *M. leprae* exposure. A diagnostic reagent, which would be analogous to, but more specific than the Mantoux skin test used for detecting exposure to *M. tuberculosis*, is required. Although the Mantoux test can be positive following exposure to non-tuberculous mycobacteria, individuals with skin test indurations greater than 10 or 15 mm are at higher risk of developing tuberculosis. Such individuals can then be referred for a chest X-ray and clinical examination to confirm or exclude tuberculosis. If such a test were to be developed for leprosy it would aid in the understanding of immune responses in healthy *M. leprae*-exposed individuals and those who are subclinically infected. Such a test may also be able to identify areas with greater leprosy endemicity within a country.

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Skin tests using Lepromin and Leprosin have been used, which are prepared from whole autoclaved *M. leprae* and soluble antigens from fractionated *M. leprae*, respectively. Lepromin is a biphasic test developed by Mitsuda called the Fernandez reaction [6] with reactions read after 48–72 h and also read at 4 weeks, the Mitsuda reaction. Lepromin and Leprosin are not diagnostic reagents for leprosy because they lack the required specificity and have been shown to induce positive skin test reactions in the majority of healthy endemic and non-endemic controls. The Lepromin test is better used for classifying leprosy patients across the spectrum of disease.

In order to develop a specific immunological tool for the purpose of diagnosing *M. leprae* exposure, one approach is to identify T cell epitopes within an antigen that are recognized specifically by individuals exposed to *M. leprae* and not other mycobacteria. A number of antigens have been identified that induce T cell responses from tuberculoid patients and leprosy patient contacts *in vitro*: for example, the 70-kD, 65-kD, *35*-kD, 30/31-kD, 18-kD and 10-kD antigens [7–12]. However, the majority of these antigens induce cross-reactive immune responses as there are homologues present in other mycobacterial species [13]. Previous studies using *M. leprae* fractionated antigens on nitrocellulose indicated that many additional proteins might be recognized as antigens [14–17]; however, there are problems with the specificity issue when considering such fractions as antigens.

A number of other studies have demonstrated the usefulness of *in vitro* assays for T cell proliferation or IFN-γ secretion as good correlates of skin test responsiveness in humans [18] [19]. To our knowledge, no *M. leprae* peptides to date have been used as diagnostic reagents in humans under *in vivo* conditions, although it should be possible to elicit DTH responses to such peptides in man, as shown for two human immunodeficiency virus peptides in a study on human volunteers [20]. Previous studies have demonstrated skin test responses to peptides of *M. tuberculosis* 19-kD in mice [21] or *M. leprae* 65-kD, 28-kD, 18-kD peptides in guinea pigs [22] and *M. tuberculosis* and *M. intracellulare* 19-kD peptides in guinea pigs [23]. Recently a study on bovine tuberculosis demonstrated the importance of antigen-specific cytokine readout systems for the early identification of *M. bovis* infection in cattle and stressed the potential of using antigen cocktails for immunodiagnosis [24]. Another study [25] has demonstrated the usefulness of peptide mixtures from ESAT-6 and CFP-10 as potential diagnostic reagents for tuberculosis.

In this study, IFN-γ production in response to PPD, *M. leprae* sonicate, the 45-kD antigen and 45-kD peptides was used to measure the cellular immunity of healthy, non-exposed UK controls and tuberculoid leprosy patients. The gene encoding the *M. leprae* 45-kD antigen was identified originally in a lambda gt 11 library [26,27]. Sera from tuberculoid and lepromatous leprosy patients contained antibodies reactive to the 45-kD fusion protein, but no responses were detected in sera from endemic controls or tuberculosis patients [27]. In a study in Mexico the 45-kD antigen was recognized more strongly by PBMCs from tuberculoid leprosy patients and leprosy contacts than by endemic controls, and was not recognized by T cells from tuberculosis patients [28]. This suggested that the 45-kD antigen might be specific for *M. leprae*.

Our study involved using individual peptides encompassing the entire 45-kD antigen in a standard *in vitro* PBMC assay in order to measure IFN-γ, which is an important cytokine in the protective immune response against *M. leprae*. Skin lesions from tuberculoid leprosy patients contain the type 1 cytokine IFN-γ mRNA, which is not found in lesions from lepromatous leprosy patients [29]. Lepromatous leprosy patients were not included in this study as they are unable to make *M. leprae*-specific immune responses resulting in the uncontrolled growth of the organism *in vivo* [30,31]; however, they produce immune responses to cross-reactive antigens such as PPD. In a study by Kaplan *et al*. bacterial loads were reduced significantly within the lesions of lepromatous leprosy patients when injected with recombinant IFN-γ [32]. The aim of our study was to identify immunodominant T cell determinants within the 45-kD antigen and to assess if there were differences in the IFN-γ responses from peripheral blood mononuclear cells of tuberculoid leprosy patients, healthy UK controls and tuberculosis patients to the peptides. Our study excluded LL leprosy patients, as they have depressed type 1 immune responses and defective CMI. In initial studies, all the peptides encompassing the 45-kD antigen were tested; further experiments involved testing a few peptides in additional UK controls and leprosy patients and tuberculosis patients from the endemic country to evaluate specificity. Multi-drug therapy has been highly successful in the treatment of leprosy; however, the identification of T cell epitopes that are specific to *M. leprae* could still prove of major importance in the context of identifying people who are exposed and possibly subclinically infected with *M. leprae*.

MATERIALS AND METHODS

Patients and controls

In the United Kingdom, 40 heparinized buffy coat blood bank samples (North London Blood Transfusion Service, Colindale, UK and South London Blood Transfusion Service, Tooting, UK) were used as a source of PBMC from leprosy-unexposed individuals. As the blood bank will not accept blood donations from volunteers who may have lived or spent long periods in the tropics, the blood used was derived from individuals who would not have been exposed to *M. leprae*. Blood samples from six pulmonary tuberculosis patients in the United Kingdom were obtained with informed consent, recruited at the Homerton Hospital, London. All patients were on antituberculosis therapy and only two were of Caucasian origin, the rest were from Africa or Indian subcontinent.

In Pakistan, blood samples were obtained with informed consent from 31 paucibacillary leprosy patients (polar tuberculoid/borderline tuberculoid TT/BT). The leprosy patients were all of Pakistani ethnic origin and recruited at the Marie Adelaide Leprosy Centre, Karachi, Pakistan. Patients were diagnosed and classified by standard clinical and bacteriological parameters and all had BCG scars. All the patients were in the early stages of multi drug therapy (less than 4 weeks' treatment) and none had a previous history of tuberculosis or other diseases. Sixteen pulmonary tuberculosis patients were recruited at Masoomeen Hospital, Karachi; these patients were on standard antituberculosis treatment.

Reagents

Sixty-seven 17-mer *s*ynthetic peptides spanning the sequence of the *M. leprae* 45 kD antigen, overlapping by 10 amino acids, were synthesized with long-chain biotin linked to the peptide by a

spacer. The synthetic peptides were synthesized using Fmoc chemistry methodology and their purity was confirmed by reverse-phase HPLC. A selection of the peptides were screened for endotoxin contamination; endotoxin contamination was 0·08–0·4 endotoxin unit per ml (EU/ml), giving a final concentration in the assays of 0·008–0·04 EU/ml. There was no correlation between the frequency of responders to peptides with 0·04 EU/ml or 0·008 EU/ml of endotoxin. The programs FINDPATTERNS and SYFPEITHI were used to predict the presence of HLA-DR binding motifs in the peptide sequences (http://134·2.96·221/Scripts/MHCServer. dLl/EpPredict.htm). In the United Kingdom, 67 overlapping peptides were used to test for T cell responses. In Karachi, because of the smaller volumes of blood available from patients, 40 peptides were tested initially (every second peptide out of the total 67, but still encompassing the whole 45-kD antigen) and then three peptides tested in subsequent experiments. The peptides were used at a final concentration of 10 μg/ml. The amino acid sequence of the 45 kD antigen is found on the SWISSPROT database using the accession number Q07297. *M. leprae* 45-kD was synthesized in Leiden by subcloning the gene into a pTrcHisB vector. The expressed protein was purified under denaturing conditions using a nickel chelate affinity resin and the purified protein fractions were analysed against PBS [28]. The purity of *M. leprae* 45-kD was confirmed by Coomassie blue-stained SDS-PAGE and visible contamination of *Escherichia coli* proteins was excluded by immunoblotting using a peroxidase-labelled rabbit anti-*E. coli* antiserum [28]. *M. leprae* sonicate (batch no. CD212) was obtained from Dr R. Rees, National Institute for Medical Research, Mill Hill, UK and used at a final concentration of 10 μg/ml. Phytohaemagglutinin (PHA; Difco Laboratories, West Mosley, UK) was used at a final concentration of 5 μg/ml as a positive control. *M. tuberculosis* protein purified derivative (PPD; batch no. RT 49, Statens SerumInstitut, Copenhagen, Denmark) was used at a final concentration of 10 μg/ml.

Isolation of PBMC from whole blood, lymphocyte transformation assays and cytokine assays

These were standard procedures as described in our previous study [33].

Positive control samples were included on each ELISA plate; these were pooled supernatants from PHA-stimulated cultures, which had been aliquoted and frozen until the day of the ELISA. Test samples from negative control cultures were below the detection limit of the ELISA and so subtraction of background cytokine measurement from test wells was not necessary. The mitogen PHA was used as a positive control: all the subjects were responders (IFN-γ production ≥100 pg/ml) to PHA.

Short-term cell lines

PBMCs obtained from normal healthy donors were isolated from heparinized venous blood by density-gradient sedimentation over Ficoll as described above. Adherent cells (AC) were cultured over 14 days and non-adherent cells (NAC) were stimulated with peptide p45 at 1 mg/ml for 1 h and then plated out in wells at 2×10^5 PBMCs/well. The PBMCs were fed every 3 or 4 days with 10% Lymphocult T (Biotest, Solihull, UK) and growth medium. After 2 weeks' short-term cell lines were added to washed monocyte-derived macrophages (APCs) not presenting any peptide or antigen (negative control), onto monocytes pulsed with peptide p45 at a final concentration of 10 μg/ml or placed onto monocytes pulsed with peptide p18 (from the amino terminal of the 45-kD antigen). All the cell lines were stimulated for 2–3 days at 37°C in a humidified incubator. Supernatants were removed before cell harvest for flow cytometry and stored at –20°C for subsequent ELISA testing.

Monoclonal antibodies

Antibodies to CD3 fluorescein isothiocyanate (FITC), CD25 phycoerythrin (PE) were purchased from Becton Dickinson, Cowley, Oxford, UK. Mouse IgG1 isotype control FITC and mouse IgG1 isotype control PE were purchased from Pharmingen (obtained through Becton Dickinson, Cowley, Oxford, UK).

HLA typing

Low resolution HLA typing of UK controls and Pakistani leprosy and tuberculosis patients was performed at the Hammersmith Hospital, Hammersmith, UK using PCR.

Criteria for threshold cut-offs

Threshold cut-offs were used to determine positive and negative responses in lymphocyte stimulation assays. Analysis of the data from unstimulated PBMC cultures of UK controls and leprosy patients showed that no IFN-γ was detected in these control wells (below the ELISA detection limit, 50 pg/ml). Therefore, there was no need to subtract any background IFN-γ. A value of IFN-γ greater than or equal to 100 pg/ml (≥100 pg/ml), double the detection limit of the assay was defined as a positive IFN-γ response.

Statistical analysis

A non-parametric test (Wilcoxon rank sum test, P) was used to evaluate the statistical significance for comparison of peptide responses between healthy UK donors and tuberculoid (TT/BT) leprosy patients in Pakistan. This was performed using the STATA 6 program and values of $P < 0{\cdot}05$ were considered to be significant.

RESULTS

Response to mycobacterial antigens in the study subjects

PBMC cultures from 20 UK non-exposed controls and 15 polar/borderline tuberculoid leprosy patients were stimulated with PHA, PPD, *M. leprae* sonicate and *M. leprae* 45-kD antigen for 5 days and IFN-γ measured in the culture supernatants by ELISA. The scatter plot in Fig. 1 illustrates the responses from individuals in both groups. Both groups responded strongly to PHA and *M. tuberculosis* PPD. Using production of IFN-γ (100 pg/ml to define a positive response, 87% leprosy patients responded to *M. leprae* sonicate, more strongly than the UK donors as expected (20% responders) (mean 1260 pg/ml, median 700 pg/ml, range 0–6950 pg/ml and mean 98 pg/ml, median 0 pg/ml, range 0–850 pg/ml, respectively). Both groups of donors showed very similar recognition of the 45-kD antigen (87% responders, mean 3190 pg/ml, median 1300 pg/ml, range 0–13500 pg/ml; 85% responders, mean 2465 pg/ml, median 800 pg/ml, range 0–14700 pg/ml, respectively). As most UK controls responded to *M. tuberculosis* PPD it is possible that the responses observed to *M. leprae* 45-kD antigen could be a result of prior mycobacterial exposure due to BCG vaccination. All subjects responded to the mitogen PHA.

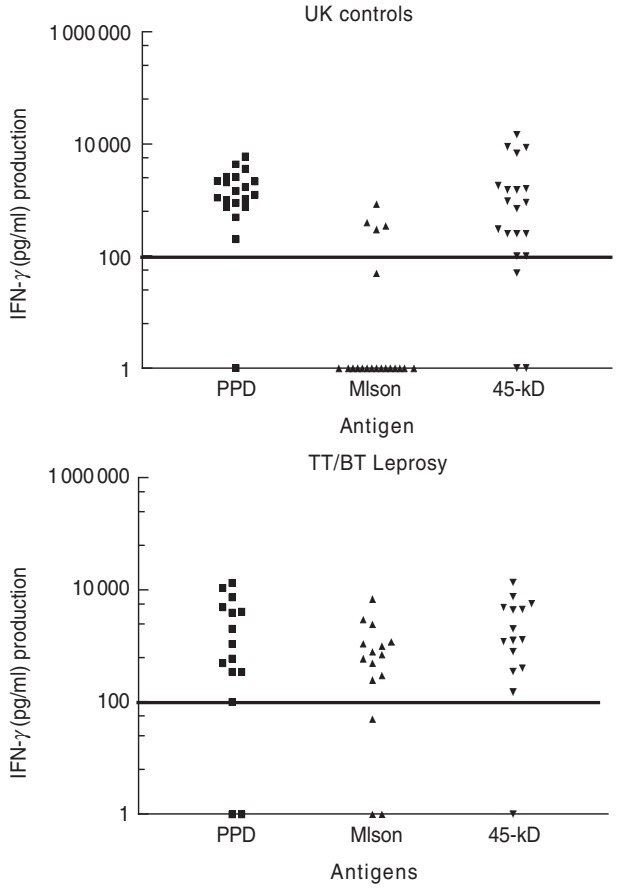

Fig. 1. IFN-γ responses induced by UK controls and TT/BT leprosy patients to *M. tuberculosis* PPD, *M. leprae* sonicate (MIson) and 45-kD antigen in the initial testing. PBMCs were stimulated for 5 days and IFN-γ was measured in the culture supernatants by ELISA. Concentrations of ≥100 pg/ml of IFN-γ (twice the detection limit of the ELISA) were used to define a positive response as shown by the line. $N = 20$ for UK controls and $n = 15$ for TT/BT leprosy patients.

IFN-γ production in response to 45 kD peptides by UK controls

PBMC from the UK non-exposed subjects were tested against 67 synthetic peptides spanning the sequence of the *M. leprae* 45-kD antigen. Certain immunodominant peptides induced frequent responses in the UK controls (Table 1). *M. leprae* 45-kD peptide sequences were compared to homologous *M. tuberculosis* predicted proteins using BLASTP on the Sanger Centre database. Sequences were selected based on those that had highest scoring segment pairs (*P*-values indicating the significance of homology). Peptides p4 and p21 had relatively few amino acid mismatches with the equivalent *M. tuberculosis* sequence (6/17, 5/17, respectively), compared to peptides p12, p23, p32, p33, p34 and p45 which had 9/17, 9/17, 11/17, 10/17, 15/17 and 10/17 amino acid mismatches, respectively (see Table 2a). Peptides p4, p32 and p45 were recognized significantly more strongly by the UK controls ($P < 0{\cdot}006$, $P < 0{\cdot}003$, $P < 0{\cdot}011$, respectively) than by leprosy patients in Pakistan. This suggests that these peptides are not *M. leprae* specific but are cross-reactive with sequences from a homologous antigen to which UK subjects have been exposed.

Table 1. A comparison of responses between tuberculoid leprosy patients and UK controls to peptides from the *M. leprae* 45-kD antigen in the initial testing

	% responders		
Peptides	TT/BT leprosy ($n = 15$)	UK controls ($n = 20$)	*P*
p4†	0%	35%	0·006
p12	0%	25%	0·151
p21	27%	35%	0·951
p23	7%	20%	0·307
p32†	0%	45%	0·003
p33	13%	35%	0·074
p34	13%	30%	0·435
p45†	20%	65%	0·011
p6	13%	5%	0·395
p10	13%	0%	0·097
p18*	13%	5%	0·066
p20*	40%	10%	0·003
p29	7%	20%	0·769

PBMCs from tuberculoid (TT/BT) leprosy patients and UK controls were stimulated with peptides and IFN-γ measured in supernatants collected at 5 days. Peptides p6–p29 were recognized more strongly (higher IFN-γ production) by T cells from the tuberculoid leprosy patients than the UK controls. A non-parametric test (Wilcoxon rank sum test) was used to evaluate the statistical significance for comparison of peptide responses between tuberculoid leprosy patients in Pakistan and UK controls (significance defined as $P < 0{\cdot}05$). †Peptides recognized to a significantly greater extent by the UK controls than the tuberculoid leprosy patients. Peptides p4–p45 were recognized more strongly (higher IFN-γ production) by T cells from the UK controls than the tuberculoid leprosy patients. *Peptides recognized to a significantly greater extent by the TT/BT leprosy patients than the UK controls.

Recognition of M. leprae *45-kD peptides by tuberculoid leprosy patients*

PBMC from leprosy patients were tested with 40 synthetic peptides spanning the entire 45-kD antigen. Some peptides were recognized preferentially by T cells from the leprosy patients rather than by UK controls, as shown in Table 1, which shows the percentage of responders in the IFN-γ assay to these peptides. Amino acid comparisons of the immunodominant *M. leprae* 45-kD antigen peptides to the equivalent predicted amino acid sequences of *M. tuberculosis* are shown in Table 2b. As can be seen from the amino acid comparisons between the two species, peptides p10 and p29 both have 11/17 amino acid mismatches with *M. tuberculosis* but of these, only peptide p10 seems to show slightly more recognition by leprosy patients than UK controls but this is not significant ($P < 0{\cdot}0976$) as shown in Table 2b. Peptides p6, p18 and p20 have slightly fewer amino acid mismatches to the corresponding *M. tuberculosis* sequences (8/17, 10/17 and 6/17 mismatches, respectively). Despite peptide p20 having only 6/17 mismatches (at positions 1, 2, 3, 5, 6 and 16) it is still a prominently recognized peptide within tuberculoid leprosy patients, with greater recognition than in the UK controls (40% and 10% responders, respectively, $P < 0{\cdot}0030$) (Table 1). Peptide p18 was also more often recognized significantly by the leprosy patients ($P < 0{\cdot}066$); however, the value is not within the signifi-

Table 2. Amino acid sequence comparisons between *Mycobacterium leprae* 45-kD peptides and homologueues in *M. tuberculosis*:

		P
(a) Peptides recognized more strongly by UK controls than by leprosy patients		
Peptide p4†		
M. leprae 45-kD	GPGS**T**P**LWG**AA**E**AW**I**SL	0·074
M. tuberculosis Rv2892c (PPE)	GPGS**GP**M**MA**AA**A**AW**D**SL	
Peptide p12		
M. leprae 45-kD	ASR**VSTF** V**A**W**LD** G**N**A**EN**	0·94
M tuberculosis Rv0611c (hypothetical protein MW13621)	ASR**QSSM**V**S** W**NH**G**A**A**GW**	
Peptide p21		
M. leprae 45-kD	LKAINWFGQ**V**ST**TV**A**AL**	0·0047
M. tuberculosis Rv2108 (PPE)	LKAINWFGQ**F**ST**RI**A**DK**	
Peptide p23		
M. leprae 45-kD	**TV**A**AL**EADY**DL** MW**V**Q**NS**	0·36
M. tuberculosis Rv2108 (PPE)	**RI**A**D K**EADY**EQ**MW**F**Q**DA**	
Peptide p32†		
M. leprae 45-kD	V**NS**FD**SSSSS**D**SLY**E**SI**	0·999
M. tuberculosis Rv3597c (lsr2)	V**DD**FD**G**S**GAA**D***ETV***E**FG**	
Peptide p33		
M. leprae 45-kD	**S**SS**S**D**SLY**ES**I**D**N**L**YDS**	0·87
M. tuberculosis Rv0027 (hypothetical protein MW 1191)	**P**SS**H**D**AIR**ES**L**D**S**L**GPI**	
Peptide p34		
M. leprae 45-kD	**LY**E***SI***D**NLYDSVAQSEE**	0·81
M. tuberculosis Rv3073c (hypothetical protein MW 13751)	**QE**E**LH**D**SAALAELRKLT**	
Peptide p45†		
M. leprae 45-kD	L**DSI I SSAS**ASLLT**TNS**	0·992
M. tuberculosis Rv0164 (hypothetical protein MW20166)	L**F**S**VVATGA**ASLLT	
(b) Peptides recognized more strongly by leprosy patients than by UK controls		
Peptide p6		
M. leprae 45 kD	AW**I**SL**AEQ**L**MEA**A**Q**EVS	0·18
M. tuberculosis Rv3746c (PE)	AW**V**SL**SSL**L**PAG**A**E**EVS	
Peptide p10		
M. leprae 45 kD	P**A**SF**A**G**ETS**D**M**L**ASRVS**	0·992
M. tuberculosis Rv2829c (hypothetical protein MW 14611)	P**S** SF**P**G**DPA**D**R**L**IYATA**	
Peptide p18*		
M. leprae 45 kD	**AGMV**PL**L**T**VL**GN**I**IH**TM**	0·995
M. tuberculosis Rv2466c (hypothetical protein MW 23036)	**KVLD**PL**Y**T**AM**GN**R**IH**NQ**	
Peptide p20*		
M. leprae 45 kD	**I I H**T**MA**LKAINWFGQ**V**S	0·013
M. tuberculosis Rv2108 (PPE)	**RVQ**T**TV**LKAINWFGQ**F**S	
Peptide p29		
M. leprae 45 kD	**FEPAPQ**L**V S**R**Y**C**MD**RRD	0·83
M. tuberculosis Rv0847 (lpqS)	**VRTGQD**L**L T** R**F** C**L A**RR-	

Peptides p4, p12, p21, p23, p32, p33, p34 and p45 have 6, 9, 5, 9, 11, 10, 15, and 10, respectively, out of 17 amino acid mismatches with sequences from *M. tuberculosis* and induced stronger responses in UK controls compared to leprosy patients. Peptides p6, p10, p18, p20 and p29 have 8, 11, 10, 6 and 11, respectively, of 17 amino acid mismatches, induced greater responses in leprosy patients than UK controls. These amino acid comparisons were performed by performing BLASTP searches to *M. tuberculosis* predicted proteins on the Sanger Centre Database. Sequences with highest scoring segment pairs were chosen (*P*-values). Amino acids in bold and underlined are different in the two species and the information in the brackets is the gene name where known; †denotes significant differences between UK > leprosy and *denotes significant differences between leprosy > UK as determined by Wilcoxon rank sum test.

cance criteria (i.e. $P < 0{\cdot}05$). In subsequent testing peptide p19, which overlaps both the p18 and p20 sequences, was also included in the testing in order to localize the epitopes recognized more precisely.

Generation of peptide-specific short-term cell lines

To confirm that subjects were making antigen-specific T cell responses to the peptide, $CD3^+$ T cell lines (45K-1, 45K-2) were derived from two UK controls whose PBMC proliferated in response to a cross-reactive peptide from *M. leprae* 45-kD antigen (peptide p45) (data not shown). Activation of $CD3^+$ T cells in the 45K-1 short-term T cell line following peptide restimulation was measured by CD25 expression (a marker for IL-2Rα chain), indicating that peptide p45 was being recognized by antigen-specific T cells (more than half the $CD3^+$ T cells were activated, 55%). By comparison a short-term cell line without any stimulation, resulted in the loss of total $CD3^+$ T cells (66% compared to 86% in stimulated cell line); this could be attributed to cells dying

because they had not received any stimulation. Restimulation of the T cell lines with this peptide p45 resulted in secretion of IFN-γ as shown in Fig. 2.

Further specificity testing in leprosy and tuberculosis patients

In subsequent experiments, PBMC cultures from a further group of 16 polar/borderline tuberculoid leprosy patients from Pakistan, 20 non-exposed controls from the United Kingdom, six UK tuberculosis patients and 16 Pakistani tuberculosis patients were stimulated with selected peptides, PHA, PPD and *M. leprae* sonicate. Strong responses were detected to PPD by Pakistani leprosy patients (100%), UK controls (85%), UK tuberculosis patients (100%) and Pakistani tuberculosis patients (94%) with mean production of 8706 pg/ml (range 750–18000 pg/ml), 3633 pg/ml (range 0–13850 pg/ml), 10625 pg/ml (range 5300–23600 pg/ml) and 10691 pg/ml (range 0–30700 pg/ml), respectively. The leprosy patients responded more strongly (88%) to *M. leprae* sonicate with a mean IFN-γ production of 2903 pg/ml, the UK controls responded least strongly (50%, mean IFN-γ 798 pg/ml), UK tuberculosis patients gave an intermediate response (67% responders) with a mean IFN-γ production of 750 pg/ml and Pakistani tuberculosis patients responded more strongly (63%) with a mean IFN-γ production of 1597 pg/ml.

The three peptides p18, p19 and p20 were tested with PBMC from leprosy patients, UK controls and tuberculosis patients to ascertain the specificity of these peptides. Peptides p18 and p20 were chosen on the basis that they were recognized most frequently by leprosy patients and not well recognized by UK controls in the preliminary testing (20% responders) and p19 was included in subsequent testing as it had 15/17 amino acid mismatches to a homologue in *M. tuberculosis* and overlapped p18 and p20. Figure 3 shows the percentage of responders to peptides p18, p19 and p20. The leprosy patients made the strongest responses to peptides p18, p19 and p20 (50%, 63%, 56% responders, respectively) with median IFN-γ production levels of 75 pg/ml, 200 pg/ml and 225 pg/ml. None of these peptides were well recognized by the UK controls (5%, 0%, 5% responders, respectively) with ranges of only 0–200 pg/ml IFN-γ produced in response to any of the three peptides. The UK tuberculosis patients' responses to the peptides were low (17%, 0%, 33%, respectively). The peptides induced an intermediate response in the Pakistani tuberculosis patients (44%, 44%, 38%, respectively). The IFN-γ responses of individuals to peptides p18, p19 and p20 are shown in Fig. 3 illustrating the variation in recognition of individual peptides by the different groups. The six UK tuberculosis patients who came from a variety of ethnic backgrounds (three African, two UK, one Indian) recognized these peptides less well compared to the Pakistani tuberculosis patient group.

HLA type and response to individual peptides

Information on the major HLA-DR alleles expressed by the UK controls and TT/BT leprosy patients from the initial testing was available. Therefore, analysis was performed on the correlation between the presence of predicted HLA-DR binding motifs (using FINDPATTERNS and SYFPEITHI programs) in the peptides and the HLA types expressed by the subjects tested. In the case of peptide p45 (which was recognized significantly more strongly by the UK controls than the leprosy patients), there was a much higher response rate by the controls expressing the HLA-DR types to which peptide p45 was predicted to bind (for example, 10 of 11 responders to peptide p45 had DR 1, 4, 7, 1404 or 17): see Table 3. Similarly with peptide p20, 3 of 6 leprosy patients expressed DR 1, 4, 7, 1404 or 17.

The presence of the correct DR type did not predict that the individual would make a positive IFN-γ response to a particular peptide. Despite using the HLA predicting programs FINDPATTERNS and SYFPEITHI (http://134·2.96·221/scripts/MHCserver.dll/home.htm) a high proportion of the peptides seemed to show promiscuous binding by a range of different HLA alleles. For example, in the case of peptide p18 predicted to bind to DR 1, 4,

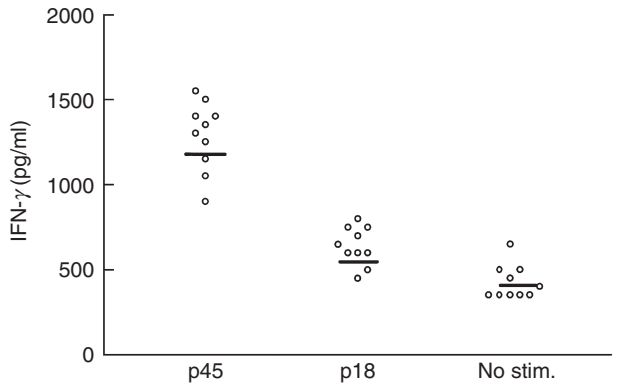

Fig. 2. The specificity of a short-term cell line for peptide p45. A short-term cell line was generated by stimulation of PBMCs with peptide p45 and fed with Lymphocult T and growth medium every 3 or 4 days. On day 14, the line was put onto macrophages presenting peptide p45 or p18 or unstimulated macrophages and cultured for 3 days. Supernatants were removed and measured for IFN-γ secretion by ELISA. Each dot represents an individual well containing a cell line, with the means represented as the horizontal lines.

Table 3. Analysing the presence of predicted HLA-DR binding motifs and HLA-DR alleles expressed by responder and non responder leprosy patients and UK controls*

Peptide predicted binding alleles and responder status	Number of subjects with alleles	Number of subjects without alleles
Leprosy		
p18 (DR 1, 4, 7, 17)		
Responder	0	2
Non-responder	8	5
p20 (DR1, 4, 7, 1404, 17)		
Responder	3	3
Non-responder	6	3
UK		
p45 (DR 1, 4, 7, 1404, 17)		
Responder	10	1
Non-responder	5	1

*Patients and UK controls were tested for their ability to secrete IFN-g in response to individual peptides as described in Materials and methods. Responders were defined as making ≥100 pg of IFN-γ per ml. HLA typing was performed by PCR. Subjects were divided into those expressing the HLA-DR alleles for which the peptide contained predicted binding motifs or lacking the correct alleles.

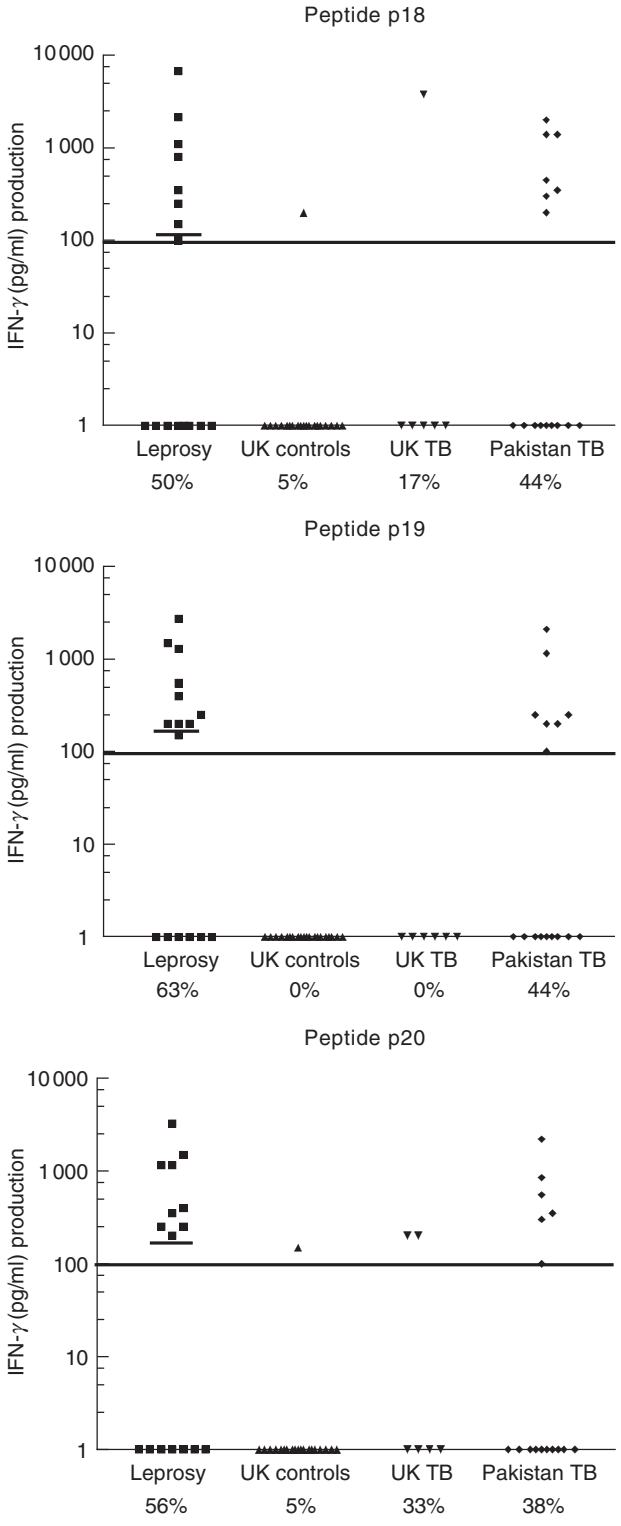

Fig. 3. IFN-γ responses induced by *M. leprae* 45-kD antigen peptides in leprosy and tuberculosis patients and UK controls. PBMCs were stimulated for 5 days and IFN-γ was measured in the culture supernatants by ELISA. Concentrations of ≥100 pg/ml of IFN-γ were used to define a positive response as shown by the line. $N = 16$ for TT/BT leprosy patients, $n = 20$ for UK controls, $n = 6$ for UK tuberculosis patients and $n = 16$ for Pakistani tuberculosis patients. Medians are indicated for the leprosy patients only, as medians for the UK controls and tuberculosis groups were zero.

7, 1404 and 17, which was recognized significantly more strongly by the TT/BT leprosy patients than the UK controls, responses were observed for the two donors who lacked these DR motifs. We are aware that the use of such prediction algorithms for class II epitopes are of limited value compared to those for class I as is seen from the data here, individuals without the correct DR type could also respond to the peptides.

From analysing results of amino acid mismatches (Sanger Centre) and DR binding (SYFPEITHI database) for the potentially *M. leprae* specific peptides p18, p19 and p20, it appears that the predicted differences between the two species are in the regions considered important for HLA binding (data not shown). For example, in peptide p18 the first three amino acid are different to the equivalent *M. tuberculosis* sequence (see Table 2b) and it is amino acids at positions 1 and 3 that are important for binding to DR 1 and DR4.

DISCUSSION

The aim of this study was to test peptides from the *M. leprae* 45-kD antigen (SWISSPROT accession number Q07297) to identify those that might be *M. leprae*-specific, which could be used as a diagnostic test reagent. Development of such a diagnostic tool would aid the monitoring of *M. leprae* transmission, which is not fully understood and also of disease distribution in endemic areas in order to target effective public health programmes to these areas. In a previous study [33] which involved identifying *M. leprae* specific peptides using the genome sequencing data, four peptides were tested that came from the 45-kD antigen, two of which showed some evidence of specificity. However, these peptides differed in sequence from those tested here.

An IFN-γ response to the 45-kD antigen could be detected in both UK controls and tuberculoid leprosy patients, although a slightly higher proportion of the leprosy patients responded. In a study in Mexico by MacFarlane *et al*. [28] 13/14 tuberculoid leprosy patients responded to the 45-kD antigen. Moreover, IFN-γ responses to 45-kD antigen were also detected in leprosy patients (68%) in Nepal [34]. This indicates that the 45-kD protein may be an immunodominant antigen. However, it also contains T cell epitopes that are recognized by T cells from UK non-exposed individuals. In a previous study we used a bioinformatics approach to identify T cell epitopes [33]; however, as we had the peptides available from a previous serology study we decided to try the conventional overlapping approach here to identify T cell epitopes. We therefore used synthetic peptides spanning the 45-kD antigen, to identify individual peptides containing T cell epitopes recognized preferentially by tuberculoid leprosy patients.

The completion of the genomes sequencing of *M. tuberculosis* and *M. Leprae* [35,36] (http://www.sanger.ac.uk) provided a unique opportunity to perform amino acid sequence comparisons of the peptides used in this study. One gene showing homology to the *M. leprae* 45-kD antigen sequence is a hypothetical 27-kD antigen coded by a PPE gene from *M. tuberculosis* (Rv2108); using the Sanger Centre database, similarities and mismatches to the *M. leprae* 45-kD peptide sequences were identified. Some individual peptides from the *M. leprae* 45-kD antigen, such as p4 and p21, had very little similarity to corresponding sequences from *M. tuberculosis*, but still induced IFN-γ responses in UK controls. One explanation for UK controls' recognition of peptides that are not similar to those of *M. tuberculosis* could be that other

environmental mycobacteria may have similar gene sequences encoding similar cross-reactive antigens. The comparisons made so far using the EBI database showed that any homology present between *M. leprae* 45-kD and other gene sequences was with gene sequences of mycobacterial species.

During initial testing a few peptides thought to have potential as being specific for *M. leprae* were also identified (p18, p20). Therefore, these peptides together with peptide p19 (overlapping p18 and p20) were tested with PBMC from another 20 UK donors, 16 TT/BT leprosy patients and 22 tuberculosis patients (from Pakistan and the United Kingdom). For the diagnostic test to be effective in a leprosy endemic country, where tuberculosis is also endemic, it ought not to generate any response in tuberculosis patients. However, the tuberculosis patients in Pakistan frequently recognized the selected peptides p18, p19 and p20. This means these peptides therefore lack specificity for *M. leprae* and would be likely to induce a positive response in subjects exposed to other mycobacterial species, if formulated as a diagnostic reagent.

After completion of initial peptide screening it was decided to confirm further that T cell responses were indeed being generated to the peptides *in vitro* (data not shown). Many studies use overlapping peptides in *in vitro* assays with the assumption that the recognition is from T cells, but we decided to confirm that responses to peptides in our assays were induced by $CD3^+$T cells and not $CD56^+$ cells. Although $CD56^+$ cells were found to produce some IFN-γ the majority of the cells making IFN-γ were $CD3^+$ T cells. This was in agreement with findings by Manandhar *et al*., who demonstrated that depletion of T cells from PBMCs revealed that $CD4^+$ T cells were the major but not exclusive contributors to 24 h IFN-γ production through stimulation by MLSA-LAM [37]. Using purified $CD56^+$ NK cells Garcia *et al*. showed in tuberculoid leprosy patients that IFN-γ production to *M. leprae* at 24 h was dependent on the presence of IL-18, but again IFN-γ was produced in a more sustained manner by $CD3^+$ T cells [38]. Using flow cytometry we were able to demonstrate that upon stimulation $CD3^+$T cells expressing $CD45RO^+$ memory marker increased considerably from 1 to 5 days (data not shown). This is similar to findings by Picker *et al*., who demonstrate the existence of *in vivo* memory/effector T cells upon stimulation with PMA and Ionomycin or SEA/SEB [39]. This study also showed that IFN-γ and IL-4 production is restricted exclusively to memory/effector cells in freshly isolated PBMCs and DTH skin sites. This is important, as most diagnostic test rely on the reagent being recognized by memory T cells that have been previously *M. leprae*-exposed. Immunohistochemical techniques have demonstrated that $CD3^+$ T cells are mainly responsible for IFN-γ in tuberculin-induced DTH skin sites [40]. In order to establish the presence of antigen-specific T cells a short-term cell line (STCL) was generated to peptide p45 from a donor. This would have the advantage of clearly 'amplifying' the detection of any peptide specific T cells present in the assay and demonstrating that PBMC responses generated to peptides are mainly the result of specific TCR recognition of peptide rather than the non-specific stimulation of NK cells. The STCL data in our study indicated clearly that T cells were activated and that such T cells were specific for the autologous peptide.

When considering the generation of a diagnostic test reagent it is important to consider the HLA class II restriction of the peptides that are to be incorporated. Ideally, it would be best to design a cocktail of peptides with the most common HLA-DR restricted binding motifs that are present in the majority of a population. It could also be possible to design a diagnostic test reagent incorporating promiscuous peptide epitopes that bind to a variety of HLA grooves but are sequence specific for *M. leprae* alone. We are aware that in using prediction algorithms there are difficulties for class II epitopes due to the degeneracy of these molecules compared to class I. However, it was interesting to observe that predicted differences in *M. leprae* specific peptides were in regions considered important for HLA binding.

It was interesting that comparison of amino acid mismatches between equivalent *M. leprae* and *M. tuberculosis* peptide sequences did not necessarily provide an indicator of specificity. This was clearly observed when even though high amino acid mismatches were present between the equivalent peptides of the two species, positive responses could still be detected in tuberculosis patients and UK controls. This may result from cross-reactive T cell responses generated as a result of prior exposure to mycobacterial antigens homologous to the *M. leprae* 45-kD antigen, such as the hypothetical *M. tuberculosis* 27-kD. Alternatively, there may be a protein present in a common environmental mycobacterial species such as *M. avium*. Comparison of the *M. leprae* 45-kD sequence with contigs of *M. avium* resulted in a match from contig 310 using the TIGR database (http://www.tigr.org), however, as the genome of this species is incomplete it cannot be assumed to be entirely accurate. This issue will be clarified as the sequencing of additional mycobacterial species is completed. UK controls may have very little exposure to *M. tuberculosis* whereas within leprosy endemic countries, the prevalence of tuberculosis is often much higher and such peptides would therefore not be suitable as a diagnostic tool. With the completion of the *M. leprae* genome project [37] it may, however, be possible to identify whole genes that are present in *M. leprae* and not in *M. tuberculosis*, and which might contain peptide epitopes with greater specificity for *M. leprae*.

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