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T cell responses to a mixture of *Mycobacterium tuberculosis* peptides with complementary HLA-DR binding profiles

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SUMMARY

The T cell response to a mixture of eight peptides derived from sequences of the *Mycobacterium tuberculosis* 16-, 19- and 38-kD antigens (MTBmix-8) has been studied. The peptides were selected on the basis of complementary binding to nine HLA-DR molecules (HLA-DR1 to DR9). MTBmix-8 at 6·25 and 50 μg/ml gave rise to significant stimulation ($P < 0{\cdot}05$) of peripheral blood mononuclear cells (PBMC) from healthy tuberculin-positive and both untreated and treated diseased subjects, but not in any of a control group of healthy tuberculin-negative subjects. MTB-mix-8 stimulated proliferation of PBMC from healthy tuberculin-positive individuals at lower concentrations than the individual component peptides. However, the maximal stimulation achieved was only slightly higher than that achieved with individual peptides. MTBmix-8 also stimulated the production of interferon-gamma (IFN-γ) *in vitro*. Using the mean $\pm$ 2 s.d. of the values for IFN-γ production in the tuberculin-negative population as a cut-off, MTBmix-8 at 6·25 μg/ml was able to detect infection with a sensitivity of 100% in untreated patients, 87% in treated patients, and 82% in tuberculin-positive controls. The corresponding figures for the most potent single peptide (16p91–110) were: 66% in untreated patients, 71% in treated patients and only 42% in controls. Thus, using the IFN-γ-based assay, which has the additional advantages of speed and does not require radioactivity, the mixture of peptides is more sensitive than single peptides in diagnosing infection.

Keywords *Mycobacterium tuberculosis* T cells peptide HLA-DR interferon-gamma

INTRODUCTION

Infection by *Mycobacterium tuberculosis* is of increased public health concern following recent moderate increases in the number of cases in developed countries [1]. The method for detection of infection is the skin reaction to purified protein derivative (PPD) of *M. tuberculosis.* Although attempts have been made to define species-specific PPD reagents, the test remains compromised in both specificity and sensitivity [2]. One of the reasons for the lack of specificity is sensitization by environmental mycobacteria [3]. This is particularly relevant in low prevalence countries which do not routinely administer bacille Calmette–Guérin (BCG) vaccination and in which control therefore is based upon the detection of infected cases and chemoprophylaxis. False-positive results may lead not only to inappropriate chemoprophylaxis but also to an overestimate of the incidence of tuberculosis within the community. In addition, there are operational problems with PPD because of its poorly defined content, batch to batch variation, difficulty in standardization, dosage and administration.

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Many of the antigenic constituents of mycobacteria are shared between human pathogens and environmental, non-pathogenic organisms. The search for alternatives to PPD has been mainly at the level of species-specific antigens. There are several candidate antigens, but none has yet been subjected to clinical trial [4–6]. An alternative to proteins would be to use peptides, which have the practical advantage of low cost. In addition, if an antigen common to more than one mycobacterial species is selected, peptides of species-specific sequence can be chosen. Consequently, if the antigen is of compromised specificity (such as the 19 kD [7]) it is possible to select epitopes from regions of the genome which are *M. tuberculosis*-specific. In this study we have used epitopes of the 16-, 19- and 38-kD molecules of *M. tuberculosis* [8–10].

The major obstacle in the use of peptides, MHC restriction, is in part offset by genetically permissive recognition of the immunodominant epitopes [11–13]. However, immunodominant epitopes are selectively rather than universally permissive, as they often fail to bind certain alleles (Jurcevic *et al*., submitted for publication). An additional problem is presented by the low precursor frequency of T cells recognizing any single epitope (approx. 1 in 100 000). The practical consequence is that

the magnitude of the *in vitro* response is usually considerably lower to a single epitope than to a whole organism or to PPD.

The aim of this study was to explore if mixing of a limited number of *M. tuberculosis*-specific and genetically permissive immunodominant epitopes would constitute a T cell reagent of amplified test sensitivity, matching that of PPD. For this purpose, we selected eight *M. tuberculosis* peptides (MTBmix-8) on the basis of their complementary binding to a wide range of HLA-DR molecules. As MTBmix-8 contains at least one peptide which binds to each of the major HLA-DR alleles, we expected that such a mixture could achieve broad T cell recognition and hence increase the test sensitivity for human populations. Furthermore, we assumed that an individual should ideally recognize several epitopes from the MTBmix-8 which would lead to an overall increase in stimulatory capacity and amplified T cell proliferation and interferon-gamma (IFN-γ) responses.

SUBJECTS AND METHODS

Subjects

Patients were recruited from the Department of Infection and Tropical Medicine (Northwick Park Hospital, Harrow, UK). On anonymous testing, none was positive by ELISA for HIV-1 (R. Wall, personal communication). Proliferative responses and/or IFN-γ production of peripheral blood mononuclear cells (PBMC) were studied in 16 untreated patients (UP; 12 male, four female, average age 29·2 years), 20 patients during treatment (TP; 12 male, eight female, average age 38·0 years), 17 PPD^+ controls (10 male, seven female, average age 37·1 years) and six PPD^- controls (one male, five female, average age 26·5 years). Of the UP, 12 had pulmonary tuberculosis, of whom 11 were sputum-positive. The remaining four patients had lymphadenopathic ($n = 2$), mesenteric, and musculoskeletal disease. Thirteen of the treated patients had pulmonary tuberculosis, three lymphadenopathy, and one each of miliary, genitourinary, osteomyelitis, and musculoskeletal disease. Ethical approval for this study was given by the Harrow Local Research Ethical Committee, EC1646. PPD^+ healthy controls who had received BCG vaccination were recruited from laboratory and clinical colleagues. Most were of low occupational exposure to tuberculosis. PPD positivity was defined as a skin reaction of > 5 mm induration to one tuberculin unit. PPD^- healthy controls, who had not received BCG vaccine, were recruited from the psychiatric nursing staff at the Maasziekenhuis (Boxmeer, The Netherlands).

Synthetic peptides

Peptides were made using solid-phase/Fmoc chemistry. Sequence integrity was verified by mass spectrometry and homogeneity by reverse-phase high-performance liquid chromatography (HPLC). Peptide purity was > 85%. N-terminally biotinylated peptides were made by shaking the fully side-chain-protected peptide resin in a solution of 1·5 equivalents of both biotinamidocaproate-N-hydroxysuccinimide ester and diisopropylethylamine in dimethyl sulphoxide (50 ml per mmol) for 2 h; cleavage, deprotection and work up were then carried out in the usual manner. Peptide sequences are shown in Table 1. The sequence of the HA/306–18 peptide is APKYVKQNTLKLAT (an N-terminal alanine was added to the original sequence so as to facilitate biotinylation); the CLIP peptide (human invariant chain 104–119) sequence is VSKMRMATPLLMQALP. Biotinylated HA/306–18 (^{bio}HA/306–18) and CLIP were used as references in binding competition assays. Polyalanine substituted peptides were variants of the 38p350–369 peptide, sequences: AAAFQPAAAAAAKAA; AAAFQPLAAAA-AKAA and AAAFQPAAAAVAKAA.

Purification of HLA-DR molecules

HLA-DR molecules were purified essentially as described before [14,15]. Briefly, Epstein–Barr virus (EBV)-transformed HLA-DR homozygous lymphoblastoid B cell lines (LBL), HOM-2, MGAR, VAVY, BOLETH, SWEIG, AMALA, MOU, MADURA and DKB, were lysed at 10^8 cells/ml in PBS containing 1% NP-40, 0·5 mM PMSF and 0·05% NaN_3 for 2 h at 4°C. Cell debris was removed by centrifugation at 10 000 ***g*** for 1 h. HLA-DR molecules were isolated from the lysates by recirculating (0·5 ml/min) through a 5 ml column of Protein-A/Sepharose CL-4B covalently coupled to the L-243 MoAb (anti-MHC). The column was then washed with 50 ml of PBS + 10 mM *n*-octyl β-D-glucopyranoside (OG) and the HLA-DR protein eluted from the column at pH 11·5 (0·15 M NaCl, 0·05 M $NaHCO_3$, 10 mM OG): 1·0 ml fractions were collected directly into 0·25 ml of 1 M NaH_2PO_4 pH 6·8. The protein-containing fractions were pooled and concentrated using Centriprep-30 ultra-filtration membranes (Amicon Inc., Beverly, MA). The final protein concentration was determined using a Microtitre BCA assay (Pierce Chemical Co., Rockford, IL).

MHC–peptide binding assay

MHC–peptide binding was tested using purified HLA-DR molecules [14]. Briefly, HLA-DR molecules (0·5 μM), biotinylated reference peptide (1·6 μM CLIP or 1·8 μM HA/306–18) and competitor peptide (0–1000 μM) were mixed in a total volume of 30 μl of binding buffer (0·1 M sodium citrate pH 6·0 + 0·5% NP-40). After 48 h incubation at room temperature, the MHC–peptide complexes were diluted with 100 μl of blocking buffer (5% non-fat dry milk/PBS, 0·1% Tween-20), divided into triplicate sets and transferred to previously prepared microtitre plates coated overnight with L-243 MoAb (at 1 μg/well). After 2 h incubation with shaking at 4°C, plates were washed six times with PBS/0·1% Tween-20 and the quantity of bound biotinylated peptide was determined colorimetrically using Streptavidin-peroxidase and tetramethyl benzidine (Sigma Chemical Co., Poole, UK). Absorbances in the presence of the reference peptide, without added competitor, were in the range 0·2–0·5, while the background absorbances were < 0·02. Statistical analyses were performed using Student's *t*-test at the level of $P < 0·05$.

Lymphocyte proliferation test

Whole citrated blood was diluted 1:2 and PBMC were separated on a Ficoll gradient (Pharmacia, Uppsala, Sweden). The cells were then washed twice in PBS and plated in triplicate wells at $1·5 \times 10^5$/well in the presence of 5% AB + human serum in 96 U-well plates (Nunc, Roskilde, Denmark) for 6 days. DNA synthesis was assayed by ^{3}H-thymidine incorporation (Amersham, Aylesbury, UK).

Induction and assay of IFN-γ

PBMC (5×10^6/ml) were cultured for 48 h in the presence of antigen and 5% AB + human serum. Supernatants were harvested and stored at –70°C until use. Maxisorp (Nunc) plates were coated with 50 μl/well of murine anti-IFN-γ MoAb 1-DIK at 2·5 μg/ml (Mabtech AB, Stockholm, Sweden). The plate was blocked for 1 h at room temperature in PBS/0·05% Tween/3% w/v bovine serum albumin. Each plate was calibrated against a standard preparation

of cytokine (Genzyme, Cambridge, MA). The plates were incubated with 100 μl sample/well in duplicate for 4 h and then washed four times. The plates were incubated at room temperature for 1 h in 100 μl biotinylated detection antibody MoAb 7B-61 anti-IFN-γ at 1 μg/ml (Mabtech) and then washed five times. Streptavidin-peroxidase (100 μl; 1 μg/ml) was added to each well and incubated for 45 min. Six final washes were followed by 150 μl K-Blue substrate (ELISA Technologies, Lexington, KY) followed by K-Red stop solution (50 μl) when colour development was optimal.

RESULTS

Binding of MTB peptides to HLA-DR molecules

Eight peptides derived from the sequences of the 16-, 19- and 38-kD antigens were tested for binding to several purified HLA-DR molecules. The binding affinities to HLA-DR1 varied considerably (Fig. 1): strong binding ($IC_{50} < 10$ μM) was obtained for 38p1–20, 38p350–369 and 16p91–110, whereas 38p270–289 and 16p111–130 bound with moderate affinity ($IC_{50} = 10$–100 μM). Low-affinity binding was shown by the peptide 16p21–40, whereas peptides 19p11–30 and 38p305–324 failed to bind to DR1 at concentrations used in this assay. Influenza haemagglutinin peptide (HA/306–18) was used as the high-affinity binding reference peptide for DR1.

The peptides had variable binding to nine HLA-DR alleles (Table 1). Some peptides (e.g. 16p91–110 and 38p350–369) bound several HLA-DR alleles with high affinity, although both failed to bind HLA-DR3. High-affinity binding to DR3 was found only for the 38p270–289 peptide. We concluded that a combination of peptides with complementary binding to HLA-DR molecules could provide a mixture containing at least one high-affinity peptide for each DR allele. On this principle we selected peptides (MTBmix-8) for analysis of lymphocyte stimulatory capacities (Table 1).

T cell proliferation

The lymphocyte stimulatory capacity of individual peptides and combinations of two to eight peptides was studied. In a representative experiment (Fig. 2a), the most stimulatory peptides for PBMC of a healthy PPD^+ subject were 16p111–130, 38p305–324, 16p91–110 and 38p270–289. As presented in Fig. 2b, increasing the number of peptides in an arbitrary combination increased the proliferation, but this increase was not linear and stimulation was lower than the sum of the responses to individual peptides. As the mixture containing all eight peptides was the most stimulatory, it was adopted for the subsequent evaluation in larger groups.

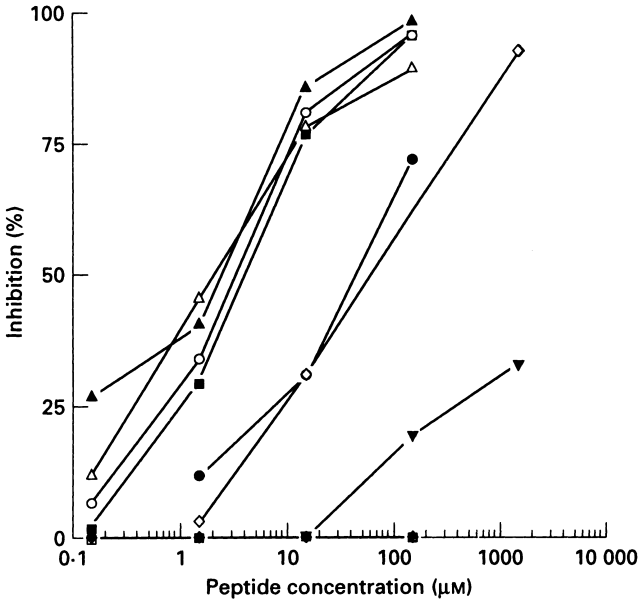

Fig. 1. Binding of *Mycobacterium tuberculosis* (MTB) peptides to the HLA-DR1 molecule. Different concentrations of *M. tuberculosis* peptides were added to compete with binding of 1·8 μM ^{bio}HA/306–18 to DR1. Absorbances in absence of inhibitor were 0·2–0·4 and backgrounds were > 0·02. ○, HA/306–18; △, 38p1–20; ◆, 38p305–324; ◇, 38p270–289; ■, 38p350–369; □, 19p11–30; ▼, 16p21–40; ▲, 16p91–110; ●, 16p111–130.

A dose–response evaluation of MTBmix-8 showed that the peptide mixture was stimulatory at lower doses than individual peptides 38p350–369 and 16p91–110 (Fig. 3). However, the plateau of stimulation achieved with MTBmix-8 was slightly higher than stimulation achieved with individual peptides. A mixture of peptides with multiple alanine substitutions, PolyAlaMix, failed to stimulate PBMC. Similar results were obtained with PBMC from five other HS controls.

By comparison with unstimulated cells, all four preparations (16p91–110 peptide, MTBmix-8 at 6·25 and 50 μg/ml and PPD) caused significant stimulation ($P < 0{\cdot}05$ in all cases) in untreated patients, treated patients, and PPD^+ controls (Fig. 4). PPD was more potent a stimulus than any peptide or peptide mix in all

Table 1. Binding of *Mycobacterium tuberculosis* (MTB) peptides to HLA-DR molecules

Peptide	Sequences	DR1	|DR2	DR3	DR4	DR5	DR6	DR7	DR8	DR9
16p21–40	LFAAFPSFAGLRPTFDTRLM	+	++	−	++	−	−	++	+	+
16p91–110	SEFAYGSFVRTVSLPVGADE	+++	+++	+	+++	+++	++	+++	+++	+++
16p11–130	DDIKATYDKGILTVSVAVSE	++	+	+	+++	−	+	+	+	++
19p11−30	GAAILVAGLSGCSSNKSTTG	−	++	−	++	−	−	−	+	+
39p1−20	MKIRLHTLLAVLTAAPLLLA	+++	+++	+	+++	−	+++	++	+++	+
39p270−289	SGNFLLPDAQSIQAAAAGFA	++	++	+++	++	++	+	++	−	+++
39p305−324	APDGYPIINYEYAIVNNRQK	−	+++	−	++	+	++	−	−	+
38p350-369	DQVHFQPLPPAVVSKDSALI	+++	++	−	+	++	+	++	+++	+

MTB peptides were tested for ability to compete binding of 1·6 μM ^{bio}CLIP to DR3 and DR9 or 1·8 μM ^{bio}HA/306−18 to other alleles. −, No binding $IC_{50} > 1000$ μM; +, low affinity $IC_{50} = 100-1000$ μM; ++, moderate affinity $IC_{50} = 10-100$ μM; +++, high affinity $IC_{50} < 10$ μM.

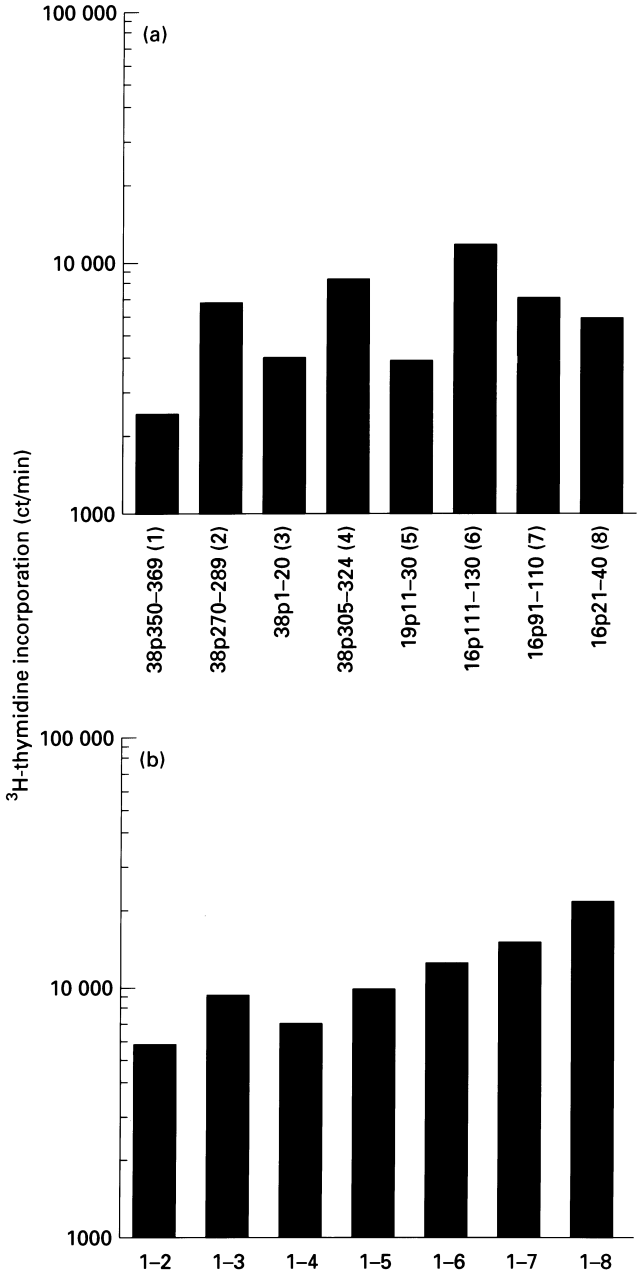

Fig. 2. Proliferative response of peripheral blood mononuclear cells (PBMC) to single *Mycobacterium tuberculosis* (MTB) peptides and their combinations. Fifty microlitres per millilitre of either individual peptides (a) or their combinations (b) were added to PBMC (10^5/well) of a purified protein derivative $(PPD)^+$ subject.

infected groups ($P < 0{\cdot}05$ in all cases). The mean value of ^{3}H-thymidine incorporation in the presence of MTBmix-8 was higher than in the presence of its constituent, 16.10 peptide, in all groups except PPD^- subjects, although the difference did not reach statistical significance. The data obtained with 16p91–110 are representative of other single peptide constituents of MTBmix–8.

IFN-γ production

A subset of the same subjects (10 UP, 16 TP, eight PPD^+ and five PPD^- controls) was studied for IFN-γ production in *ex vivo* stimulated cells (Fig. 5). In all three sensitized groups there was significantly raised IFN-γ production in response to the MTBmix-8, peptide 16p91–110 and to PPD ($P < 0{\cdot}05$) compared with medium alone. Amongst the non-sensitized controls there was no significant stimulation by any preparation. Using the mean + 2 s.d.

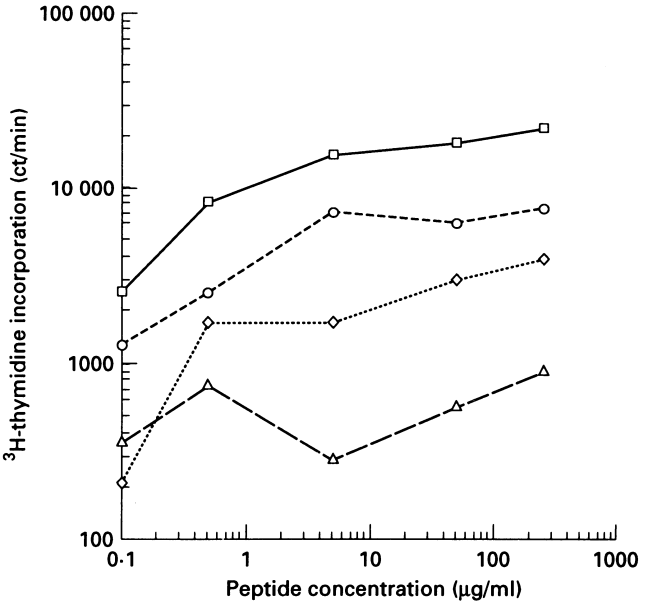

Fig. 3. Dose-dependent stimulation of peripheral blood mononuclear cells (PBMC) by single and combined *Mycobacterium tuberculosis* (MTB) peptides. Different concentrations of single MTB peptides or peptide combinations were added to PBMC (10^5/well) from a purified protein derivative $(PPD)^+$ healthy subject. The values are means of triplicates from a single, representative experiment. Variations were <20%. □, MTBmix-8; ◇, 38p350–369; ○, 16p91–110; △, PolyAlaMix.

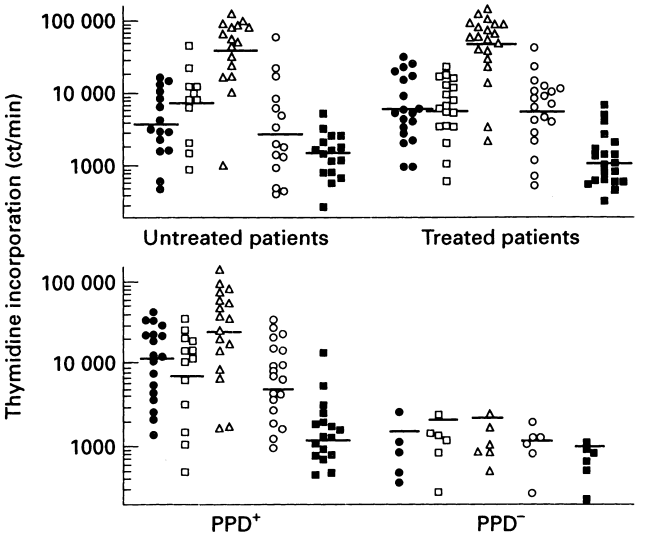

Fig. 4. Proliferation of peripheral blood mononuclear cells (PBMC) in response to peptide mixtures. Results are expressed as ^{3}H-thymidine incorporation after 6 days culture in the presence of 5% AB + serum. Horizontal bars represent the geometric mean ct/min for each preparation. ●, Mix-8 50 μg/ml of each peptide; □, mix-8 6·25 μg/ml of each peptide; △, purified protein derivative (PPD) 10 U/ml; ○, peptide 16p91–110 50 μg/ml; ■, medium alone.

of the values for the non-sensitized population as a cut-off, MTBmix-8 at 50 μg/ml was able to detect infection with a sensitivity of 89% in UP, 87% in TP, and 82% in PPD$^+$ controls. The corresponding figures for MTBmix-8 at 6·25 μg/ml were 100%, 87% and 82%, respectively; and for PPD 100% in all sensitized groups. The single peptide 16p91–110 was able to detect infection in 66% of UP, 71% of TP, and only 42% of PPD$^+$ controls. Thus, by contrast with the cell proliferative assays, in the IFN-γ-based assay the mixture of peptides is more sensitive than single peptides in diagnosing infection.

DISCUSSION

Although peptides are widely acknowledged for their potential in diagnosis and subunit vaccines [16], there have so far been very few practical applications, mainly due to the extensive polymorphism within the human MHC. Recently acquired knowledge of the MHC–peptide interaction suggests that many peptides bind permissively to several MHC molecules. However, no peptide binds universally [17]. This limitation can be overcome, as demonstrated here, by combining several peptides to complement each other in binding to a wide spectrum of human HLA alleles.

The mixture of peptides (MTBmix-8) used in this study was selected on the basis of screening of a larger set of 49 peptides derived from sequences of the 16-, 19- and 38-kD antigens for binding to HLA-DR molecules. A number of the peptides with high binding affinity to HLA-DR have been shown to cause T cell stimulation [11–13], indicating that binding to MHC and immunodominance are related. In the pilot experiments (Fig. 2b) the mixture containing eight peptides was most stimulatory and therefore adopted for the remainder of the evaluation. It is possible that further empirical testing and MHC binding data could lead to a 'better' mix.

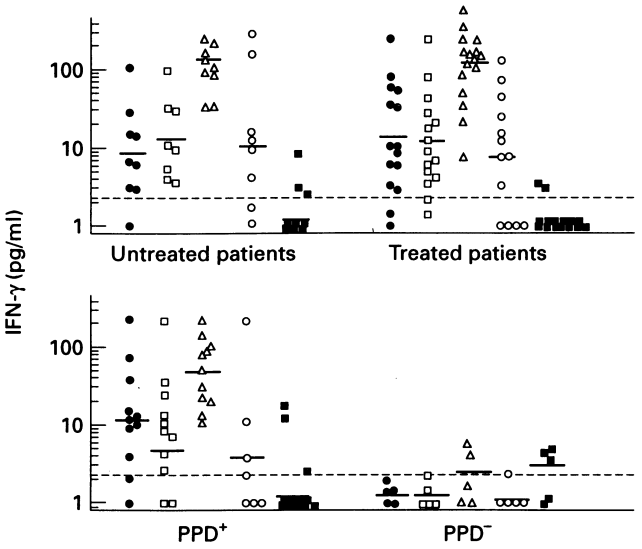

Fig. 5. IFN-γ production of peripheral blood mononuclear cells in response to peptide mixtures. Results are expressed as pg/ml after 48 h culture of 5×10^6 cells/ml in the presence of 5% AB + serum. The assay sensitivity is 1·0 pg/ml. The horizontal line indicates the cut-off for a positive assay. ●, Mix-8 50 μg/ml of each peptide; □, mix-8 6·25 μg/ml of each peptide; △, purified protein derivative (PPD) 10 U/ml; ○, peptide 16p91–110 50 μg/ml; ■, medium alone.

MTBmix-8 proved to be stimulatory in most infected or sensitized individuals and showed less individual variations than single peptides, especially in the IFN-γ-based assay (Fig. 5). Furthermore, MTBmix-8 was stimulatory at lower concentrations than single peptides: optimal concentrations for most of the single peptides were 50 μg/ml, and for MTBmix-8 it seems they were around 6·25 μg/ml of each peptide. MTBmix-8 was more efficient in stimulating the production of IFN-γ than single peptides (sensitivity of 100% in UP, 87% in TP, and 82% in PPD$^+$ controls). The broader recognition of MTBmix-8 in this assay could be explained by the fact that HLA-DR is a relative restricting element, despite permissive recognition, and that by selection of at least one peptide binding to each of the major HLA-DR types, we have overcome this obstacle.

Antigen- [18] and epitope-specific [12] anergy has been previously documented in patients with tuberculosis. When the healthy tuberculin-positive (10 321 $\pm$ 3650 ct/min) and untreated groups (3834 $\pm$ 1411 ct/min) were compared, such an effect was seen with the higher dose of MTBmix-8 ($P < 0{\cdot}01$); the same trend was seen with peptide 16p91–110, though the difference did not reach significance. Interestingly, anergy was not seen with the low-dose MTBmix-8 or PPD (Fig. 4). During treatment, these differences between peptide preparations tended to disappear, resulting in similar geometric mean ct/min in this group (Fig. 4).

Contrary to our expectations, combined peptides failed to act synergistically, or even additively, and the stimulatory capacity of MTBmix-8 was slightly higher than that of the best, single peptide (16p91–110). The absolute level of IFN-γ produced in presence of MTBmix-8 was almost invariably less than that induced by PPD. Even though several peptides from MTBmix-8 are presumably recognized by each individual, the sensitivity may still be limited by the number of epitope-specific T cells. Alternatively, this could be due to competition between peptides for a limited number of binding sites (HLA-DR molecules) on the surfaces of antigen-presenting cells (APC). Consequently, increases in either the number or concentration of peptides could lead to a decrease in the level of HLA-binding by any single peptide. This explanation is supported by the observation that MTBmix-8 in some cases had a declining stimulatory capacity at high concentrations. Thus, the peptide mixture increased the number of responders rather than their individual levels of proliferation or IFN-γ production.

Synthetic peptides are neither taken up into nor processed by APC [19] and bind to a relatively small proportion (< 10%) of MHC class II molecules on the cell surface [20]. Hence, administration of peptides in the form of multi-antigenic peptides (MAP), microspheres or liposomes could enable APC to take up the peptides and make them more readily available for loading of newly synthesized MHC molecules. In this way, competition with bound endogenous peptides could be reduced. It will be relevant to explore whether peptide modification or their incorporation into MAPs, microspheres or liposomes could improve their T cell-stimulatory capacity.

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REFERENCES

1 Dolin PJ, Raviglione MC, Kochi A. Global tuberculosis incidence and mortality during 1990–2000. Bull World Health Organ 1994; **72**:213–20.

2 Bass Jr JB. The Tuberculin test. In: Reichmann LB, Hershfield ES, eds. The Tuberculin test. New York: Marcel Dekker, 1993:139–48.

3 Grange JM. Infection and disease due to the environmental mycobacteria. Trans R Soc Trop Med Hyg 1987; **81**:179–82.

4 Harboe M, Oettinger T, Wiker HG *et al.* Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. Infect Immun 1996; **64**:16–22.

5 Ivanyi J, Thole J. Specificity and function of T and B cell recognition in tuberculosis. In: Bloom B, ed. Tuberculosis: pathogenesis, protection and control. Washington DC: American Society for Microbiology, 1994:437–58.

6 Roche PW, Triccas JA, Avery DT *et al.* Differential T cell responses to mycobacteria-secreted proteins distinguish vaccination with Bacille Calmette–Guérin from infection with *Mycobacterium tuberculosis*. J Infect Dis 1994; **170**:1326–30.

7 Harris DP, Vordermeier HM, Brett SJ *et al.* Epitope specificity and isoforms of the mycobacterial 19-kilodalton antigen. Infect Immun 1994; **62**:2963–72.

8 Ashbridge KR, Booth RJ, Watson JD *et al.* Nucleotide sequence of the 19 kDa antigen from *Mycobacterium tuberculosis*. Nucleic Acids Res 1989; **17**:1249.

9 Andersen AB, Hansen EB. Structure and mapping of antigenic domains of protein antigen b, a 38 000-molecular-weight protein of *Mycobacterium tuberculosis*. Infect Immun 1989; **57**:2481–8.

10 Verbon A, Hartskeerl RA, Schuitema A *et al.* The 14 000-molecular weight antigen of *Mycobacterium tuberculosis* is related to the alpha-crystallin family of low molecular weight heat shock proteins. J Bacteriol 1992; **174**:1352–9.

11 Faith A, Moreno C, Lathigra R *et al.* Analysis of human T-cell epitopes in the 19 000 MW antigen of *Mycobacterium tuberculosis*: influence of HLA-DR. Immunology 1991; **74**:1–7.

12 Vordermeier HM, Harris DP, Friscia G *et al.* T cell repertoire in tuberculosis: selective anergy to an immunodominant epitope of the 38 kDa antigen in patients with active disease. Eur J Immunol 1992; **22**:2631–7.

13 Friscia G, Vordermeier HM, Pasvol G *et al.* Human T cell responses to peptide epitopes of the 16 kDa antigen in tuberculosis. Clin Exp Immunol 1995; **102**:53–57.

14 Jurcevic S, Praud C, Coppin HL. Role of polymorphic residues of HLA-DR molecules on the binding of HIV peptides. Immunology 1996; **87**:414–20.

15 Buus S, Sette A, Colon SM *et al.* Isolation and characterisation of antigen–Ia complexes involved in T cell recognition. Cell 1986; **47**:1071.

16 Lanzavecchia A. Identifying strategies for immune intervention. Science 1993; **260**:937–44.

17 Sette A, Southwood S, Miller J *et al.* Binding of major histocompatibility complex class II to the invariant chain-derived peptide, CLIP, is regulated by allelic polymorphism in class II. J Exp Med 1995; **181**:677–83.

18 McMurray DN, Echeveri A. Cell-mediated immunity in anergic patients with pulmonary tuberculosis. Am Rev Resp Dis 1978; **118**:827–34.

19 Lanzavecchia A, Watts C. Peptide partners call the tune. Nature 1994; **371**:198–9.

20 O'Sullivan D, Sidney J, Appella E. Characterisation of the specificity of peptide binding to four DR haplotypes. J Immunol 1990; **145**:1799–808.