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Human T cell responses to peptide epitopes of the 16-kD antigen in tuberculosis

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SUMMARY

The 16-kD protein constituent of the *Mycobacterium tuberculosis* complex has been known mainly for its prominent serological immunogenicity and species specificity in tuberculous infection. In this study, we evaluated the T cell immune repertoire in 27 sensitized healthy subjects and 46 patients with active tuberculosis using 14 overlapping 20mer peptides spanning the entire sequence of this protein. Four of the tested peptides individually stimulated proliferation of blood mononuclear cells from more than 50% of healthy controls. Tuberculosis patients reacted to a narrower peptide range and with a 17–27% lower rate of responses to the four most immunogenic peptides, but these differences do not distinguish in any simple way between the T cell repertoire of patients and sensitized healthy subjects. The most immunogenic peptide (91–110) was recognized by 67% of healthy subjects and by 50% of tuberculosis patients. Importantly, several non-responders to this peptide were stimulated with the other three most permissive peptides with sequences of 111–130, 71–91 and 21–40, resulting in an overall response rate to at least one of these four peptides of 93% in healthy controls and 74% in tuberculosis patients. In view of this additive effect between the most immunogenic peptides, their combined use may achieve sufficient sensitivity in a test aimed at the specific discrimination between infected and non-infected healthy subjects. The major interest in testing with these peptides rests in their species specificity, which is not achieved using purified protein derivative (PPD).

Keywords tuberculosis immunodiagnosis human T cell repertoire peptides

INTRODUCTION

Following infection with tubercle bacilli, both self-healed individuals and patients with active tuberculosis have abundant T cells reactive against several mycobacterial antigens. However, it is not known whether a protective or pathogenic outcome of tuberculous infection is determined by the specificity [1] and/or by phenotypic characteristics, such as the cytokine profile, of responding T cells [2]. Previous analysis of blood mononuclear cells by *in vitro* proliferation assay to multiple synthetic peptides, derived from the 19-kD or 38-kD proteins, revealed certain epitope-specific differences between sensitized and diseased individuals [3–5]. In view of the potential of these findings for immunodiagnostic application, it is necessary to expand knowledge of the human T cell repertoire to peptide epitopes of other prominent antigens of this pathogen.

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Specificity in the diagnosis of infection with *Mycobacterium tuberculosis* using the purified protein derivative (PPD) is limited, because a positive response could indicate sensitization by environmental mycobacteria [1]. The present study has examined the human T cell repertoire to peptides of the 16-kD (originally classified as 14-kD) protein constituent of the *Myco. tuberculosis* complex. This molecule containing 144 amino acid residues has approximately 30% sequence identity with several proteins belonging to the α-crystallin family of low molecular weight heat shock proteins, including the 18-kD antigen from *Myco. leprae* [6,7]. The gene for the 16-kD protein has not been found in mycobacteria outside the *Myco. tuberculosis* complex [8], thus indicating prominent species specificity.

Studies of the antigenic structure of the 16-kD antigen using MoAbs identified one conformational epitope (TB68) and at least two linear B cell epitopes (31–40 and 60–70) specific for the *Myco. tuberculosis* complex [9]. Antibody levels to the TB68 epitope have been found elevated in about 70% of smear-positive patients with pulmonary tuberculosis, with increasing

antibody titres following chemotherapy, but low levels in particularly severe cases [10]. Antibody positivity of a proportion of sera in childhood tuberculosis [11], in tuberculosis case contacts [12], after heavy occupational exposure [13], and also in the cerebrospinal fluid of patients with tuberculous meningitis [14], suggested that selective sensitization against the 16-kD antigen occurs in the early stages of tuberculous infection.

Murine T cell responses to peptides derived from the amino acid sequence of the 16-kD antigen have been analysed using immune lymph node and spleen cells [15]. That study identified a multitude of cryptic epitopes of which only four were immunodominant and H-2 permissive. Only two of these (p21–40 and p71–91) were recognized by T cells from infected mice. Species specificity of both these epitopes was demonstrated by their failure to stimulate T cells from mice which had been primed by mycobacteria outside the *Myco. tuberculosis* complex. The purpose of the present study was to investigate the epitope specificities of the human T cell repertoire by comparing sensitized healthy subjects and patients with active disease.

PATIENTS AND METHODS

Subjects

Active tuberculosis (TB) was diagnosed in a total of 46 patients on the basis of routine clinical, bacteriological and histological parameters. Thirty patients had pulmonary disease, of whom 18 were newly diagnosed and 12 were undergoing chemotherapy for a period of between 1 and 3 months. Eight patients had lymphatic TB and eight patients had other forms of extrapulmonary TB. Microscopy of sputum or biopsy samples for acid fast staining was positive in 27 and negative in 19 patients. There was no evidence of HIV infection in any of the patients tested.

The group of sensitized healthy subjects (SH) was represented by 25 clinically healthy volunteers from laboratory staff, all of whom showed skin induration diameters of more than 10 mm at 48 h after inoculation with 10 U Tuberculin–PPD (Evans Medical Ltd, Horsham, UK). All healthy donors were bacille Calmette–Guérin (BCG) scar-positive.

Synthetic peptides

Fourteen peptides, all 20 amino acid residues long and overlapping by 10 residues, covered the complete sequence of the 16-kD protein of *Myco. tuberculosis*. Their sequences were: 1–20, MATTLPVQRHPRSLFPEFSE; 11–30, PRSLFPEFSELFAAFPSFAG; 21–40, LFAAFPSFAGLRPTFDTRLM; 31–52, LRPTFDTRLMRLEDEMKEGRYE; 41–60, RLEDEMKEGRYEVRAELPGV; 51–70, YEVRAELPGVDPDKDVDIMV; 61–80, DPDKDVDIMVRDGQLTIKAE; 71–91, RDGQLTIKAERTEQKDFDGRS; 81–100, RTEQKDFDGRSEFAYGSFVR; 91–110, SEFAYGSFVRTVSLPVGADE; 101–120, TVSLPVGADEDDIKATYDKG; 111–130, DDIKATYDKGILTVSVAVSE; 121–140, ILTVSVAVSEGPTEKHIQI; 131–144, GKPTEKHIQIRSTN.

Peptides were prepared as described previously [15] by Fmoc technology using trialcoxy-diphenyl-methylester resin and Castro's reagent for coupling procedures. Homogeneity and sequence fidelity were confirmed by analytical reverse-phase high performance liquid chromatography (HPLC), mass spectrometry and amino acid sequence determination.

Lymphocyte transformation test

Mononuclear cells freshly isolated from citrated blood by centrifugation on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) were suspended in RPMI 1640 (GIBCO, Paisley, UK) containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5% A^+ heat-inactivated human serum. Microcultures containing 2×10^5 cells/well in the presence of appropriate peptide were set up in 96-well round-bottomed microtitre plates (Nunc, Roskilde, Denmark) and incubated for 7 days at 37°C in 5% CO_2. During the last 16 h of culture, ^{3}H-thymidine (Amersham International, Aylesbury, UK), at a concentration of 18·5 kBq (= 0·5 μC) per well, was added and ^{3}H-thymidine incorporation was measured by liquid scintillation counting. Quadruplicate microcultures were regularly set up with peptides at 50 μg/ml (found as optimal concentration in preliminary experiments) as well as with Tuberculin–PPD at 100 U/ml and concanavalin A (Con A; Sigma, St Louis, MO) at 6 μg/ml as control stimulants. The s.d. values of mean radioactive counts did not exceed 15%.

Criteria of evaluation

Responses to PPD or to individual peptides were considered positive if the ct/min value of peptide-stimulated thymidine uptake was increased at least three-fold over the mean ct/min of medium control cultures. Results were expressed as Δct/min, obtained by subtraction of the mean ct/min of the medium control (generally below 500 ct/min) from the mean ct/min in peptide-containing cultures. Results are presented only from those patients and healthy subjects who were classified as responders to PPD.

RESULTS

Immunogenicity of individual peptides

Evaluation of the stimulatory hierarchy between peptides (Fig. 1a) was based on the frequency of their recognition in 25 SH subjects and 38 TB patients whose blood mononuclear cells all reacted positively to PPD *in vitro*. TB patients who failed to respond to PPD *in vitro*, also invariably failed to respond to all tested peptides and were therefore excluded from evaluation.

The most frequent responses were found to be directed against four peptides of sequences 21–40, 71–91, 91–110 and 111–130. Responses to adjacent overlapping peptides were not significantly associated, thus indicating that all tested peptides contained epitopes of distinct specificities. The hierarchy in immunogenicity was found to be similar, irrespective of the total number of stimulatory peptides (results not shown). Consequently, the less frequently stimulatory peptides were recognized mainly by individuals who responded to multiple peptides. None of these individual variations was associated with the organ localization of disease or any other clinical feature of patients.

The geometric means of Δct/min for each tested peptide were calculated from individual values obtained only in responders to the respective peptides (see numerical values above bars in Fig. 1a). These differences in mean Δct/min values, reflecting probably the stimulatory qualities of individual peptides, varied irrespective of the frequency of responders. When comparing the differences between SH and

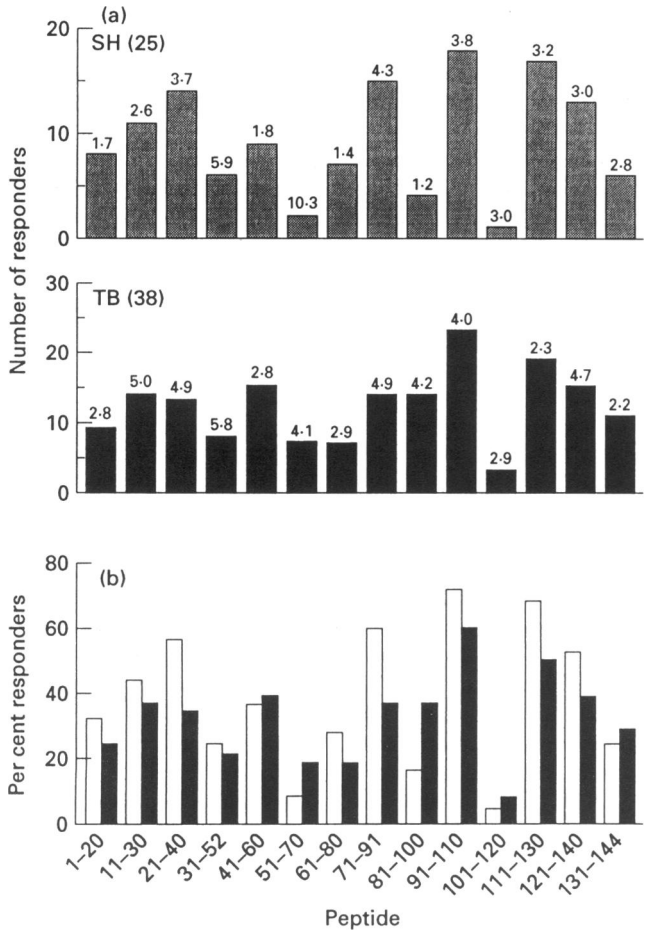

Fig. 1. Frequency of responder individuals to overlapping peptides derived from the 16-kD antigen of *Mycobacterium tuberculosis*. Responders had at least three-fold elevated thymidine uptake in lymphocyte cultures containing peptide over medium alone. (a) Absolute numbers (bars) and geometric mean values of Δct/min $\times 10^{-3}$ of responders (numbers above bars). (b) Relative numbers of responders. SH, Sensitized healthy subjects (□); TB, patients with active tuberculosis (■).

TB groups, only the increased Δct/min to p11–30 in TB patients (i.e. 5·0 *versus* $2{\cdot}6 \times 10^{-3}$ ct/min) was statistically significant (*t*-test $P < 0{\cdot}003$).

The stimulatory potencies of peptides have been compared between SH and TB groups on the basis of the relative proportion of responders to each of the 14 tested peptides (Fig. 1b). Peptide 91–110 produced the highest frequency of stimulation, in both SH and TB groups, followed closely by responses to three peptides p21–40, p71–91, p111–130. While the hierarchy of stimulatory potencies for these most immunogenic peptides was about the same in the two groups, TB responders were less frequent compared with the SH group. In contrast, it was surprising to find that responsiveness to several of the least frequently stimulatory peptides in healthy subjects became elevated in TB patients: a doubling in the number of TB responders was observed for peptides 51–70, 81–100 and 101–120. However, no consistent differences in peptide responses were found between smear-positive or smear-negative pulmonary and extrapulmonary TB (results not shown), although the small number of tested patients does not permit us to make firm conclusions.

Table 1. Frequency of peptide-reactive individuals*

Number of peptides tested positive		SH ($n = 27$) No.	SH ($n = 27$) %	TB ($n = 46$) No.	TB ($n = 46$) %
14	0	2	7·4	8	17·4
	1–3	6	22·2	20	43·5
	4–6	13	48·1	11	23·9
	>6	6	22·3	7	15·2
	At least 1	25	92·6	38	82·6
4†	0	2	7·4	12	26·1
	1	1	3·7	14	30·4
	2–3	20	74·1	15	32·6
	4	4	14·8	5	10·9
	At least 1	25	92·6	34	73·9

* Ct/min values elevated three-fold above background.
† Peptides 21–40, 71–91, 91–110, 111–130.
The difference between sensitized healthy subject (SH) and TB groups when using 14 or 4 peptides by χ^2-test; $P < 0{\cdot}01$.

Multiplicity of recognized peptides

The total number of stimulatory peptides varied from one to 13 between individuals from both SH and TB groups. We evaluated this individual variation by expressing the frequency of responders in respect of arbitrarily set numbers (1–3, 4–6 or > 6) of peptides tested (Table 1). Responders to < 4 peptides increased from 22·2% in the SH group to 43·5% in the TB group. This difference is significant (χ^2 test, $P < 0{\cdot}01$) and suggests a narrowing of the T cell repertoire in the TB group. A similar narrowing of the T cell repertoire was demonstrable also in respect of the four most immunogenic epitopes as reflected by the increase in the rate of non-responders or responders to only one of four tested peptides from 11·1% in the SH group to 56% in the TB group (χ^2 test, $P < 0{\cdot}001$). Overall, no significant association of the profile of recognized peptides with the sex, age or ethnic background of individuals was found.

Prevalence of peptide responses in SH and TB groups

It was of interest to evaluate the prospects for the possible application of peptides for the identification of infected healthy subjects and for the discrimination between infected and diseased individuals. Of the 27 PPD-positive SH subjects, 25 (92·6%) were found to be positive to at least one of the four selected immunodominant peptides (Table 1, Fig. 2). In fact, all 25 SH subjects were positively detected also when omitting p21–40, and thus reducing the number of test peptides to three. Two subjects were negative when the whole set of 14 peptides was taken into account. Of the 46 PPD-positive TB patients, only 34 (73·9%) were positive to one of the four selected peptides, but 38 (82·6%) were positive when taking account of all 14 peptides, eight (17·4%) patients failed to respond to any of the tested peptides, whilst giving undiminished proliferation to PPD. Although single peptide responsiveness to the four selected peptides was similar to that when all 14

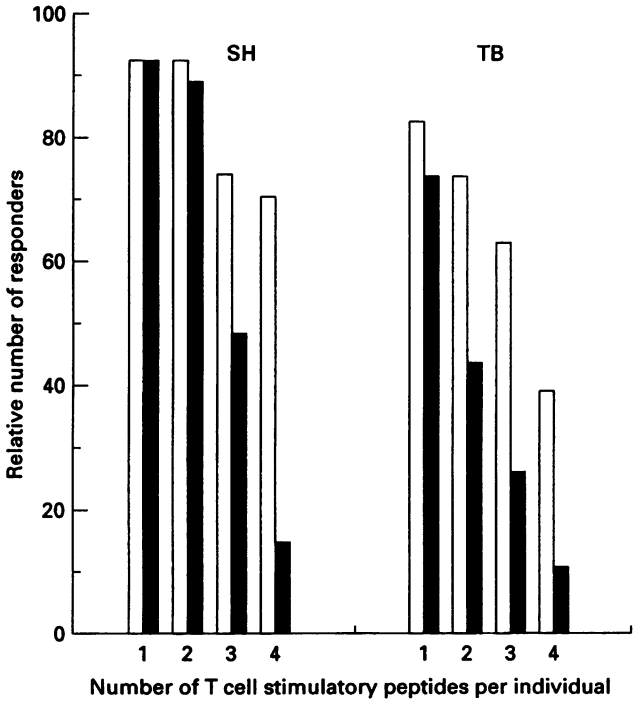

Fig. 2. Frequency of responders in relation to the number of stimulatory peptides. SH, Sensitized healthy subjects; TB, patients with active tuberculosis. Evaluation of the relative numbers of responders, established on the basis of proliferation using a total of either 14 peptides (□), or the selected four (21–40, 71–91, 91–110 and 111–130) most immunogenic peptides (■). The results suggest that a 93% positivity rate for infection, based on proliferation against at least one peptide, can be obtained using only the selected four, rather than all 14 peptides.

peptides were considered, the latter produced clearly more frequent multiple peptide responses (Fig. 2). It is of interest that multipeptide SH responses were due mainly to the most immunogenic epitopes, whereas repertoire spreading in the TB group involved some of the least immunogenic 'minor' epitopes.

Responder frequencies to the four best peptides varied between 55% and 66% (Table 2). Hence it was important to establish whether individual responsiveness was overlapping or complementary between the peptides. The results showed that more than 50% of the SH non-responders to the 'best' 91–110 peptide could be stimulated with at least one of the three immunodominant peptides (Table 2). In the TB group, peptide 111–130 had the best complementary ranking. These results clearly suggest an additive effect of testing with selected immunodominant epitopes.

Table 2. Ranking of the four most immunogenic peptides

	Proportion of responders from					
	Total number of subjects				p91–110-negative subjects	
	SH (27)*		TB (46)			
Peptide sequence	No.	%	No.	%	SH (9) No.	TB (23) No.
91–110	18	66·7	23	50·0	NA	NA
111–130	17	63·0	19	41·3	6	10
71–91	15	55·5	14	30·4	6	3
21–40	15	55·5	13	28·3	5	3
Other†	0	0	4	8·7	0	4

* Number of tested individuals.
† In non-responders to the listed four peptides.
NA, Not applicable.

DISCUSSION

The presented analysis of a complete set of overlapping peptides of the 16-kD antigen identified four peptides which stimulated the blood mononuclear cells of more than 50% of healthy sensitized subjects. Judging from such high frequency of responders in a randomly chosen population, these peptides are likely to be stimulatory for individuals with diverse HLA haplotypes, i.e. genetically permissive. The observed association between immunodominance and apparent genetic permissiveness of mycobacterial epitopes is in accord with results from the previously reported analysis of human responses to peptides derived from the sequence of the 19-kD and 38-kD antigens of *Myco. tuberculosis* [3–5].

The results of this study showed a striking variation in the number of stimulatory peptides between respective individuals. Multiple peptides were more often stimulatory in healthy sensitized subjects, whereas the responses of a significant number of patients with active disease became narrowed to a fewer number of the most immunogenic peptides. Therefore, responsiveness to multiple peptides is most likely to result from a generally higher frequency of primed T cells. It will be of interest to investigate sensitized family-related individuals to determine whether genetic factors influence the multi-epitopic and pauci-epitopic patterns of T cell recognition.

Comparison of the human T cell repertoire to epitopes of the 16-kD antigen with that previously reported in mice [15] showed several interesting features. The human repertoire was found to be wider, including frequent recognition of two epitopes (91–110 and 121–140) which are cryptic in mice. There was a narrowing in the number of stimulatory peptides in TB compared with SH subjects, but less striking than in *Myco. tuberculosis*-infected C57Bl/10 mice, which recognized only two epitopes (p21–40 and p71–91). Nevertheless, both these peptides were also among the most frequently recognized in humans, and remain of potential diagnostic importance in view of their specificity for the *Myco. tuberculosis* complex, including the lack of response to sensitization with *Myco. avium-intracellulare* [15]. The most active peptide in humans, p91–110, had previously been characterized as a cryptic epitope in mice of both H-2^b and H-2^k haplotype [15], whereas p111–130 was immunodominant in both mice and humans. Changes in the nature of epitopes from cryptic to immunodominant have been proposed as a possible basis for autoimmune disease [16], although its mechanisms are not understood. However, we cannot judge whether these differences could contribute to the pathogenic consequences of tuberculous infection without

ascertaining the cytokine-secreting profiles of the epitope-specific T cells.

Peptides which were found to be stimulatory in only a small fraction of individuals (e.g. 51–70 or 81–100) are the likely candidates for epitopes which are recognized by certain HLA haplotypes. Interestingly, responsiveness to these 'minor' epitopes was more frequent in TB patients than in SH subjects, which is the opposite trend compared with the frequently recognized epitopes. It will be of further interest to investigate whether the higher frequency of responsiveness to certain peptides could be associated with a skewed frequency of any HLA-DR or DQ haplotype specificities in patients with tuberculosis [17].

An important outcome of this study relates to the prospects of peptide-based detection of T cell sensitization following tuberculous infection. Although none of the tested single peptides reached a sufficiently high level of detection sensitivity, we demonstrated that a combination of four or even three selected peptides could positively detect 25 out of 27 (i.e. 92·6%) sensitized healthy subjects. This outcome resulted from the additive effect between the four selected relatively permissive epitopes. This complementarity and the known species-specificity of epitopes of the 16-kD antigen are encouraging for the prospects of developing an optimal combination of peptides of equal sensitivity but superior specificity to PPD.

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