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Recognition of peptide epitopes of the 16,000 MW antigen of *Mycobacterium tuberculosis* by murine T cells

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SUMMARY

The T-cell repertoire to a prominent immunogen of *Mycobacterium tuberculosis* has been investigated on the assumption that differences in epitope specificity could influence the protective and pathogenic host reactions. Proliferative responses of lymph node and spleen cells to overlapping peptides, spanning the entire sequence of the 16,000 MW protein antigen were analysed in C57BL/10 and B10.BR mice. Following footpad priming and *in vitro* challenge with homologous peptide, 12 out of the 14 peptides tested were found to be immunogenic. However, only two peptides of residues 31–40 and 71–91 stimulated strong proliferative responses of T cells from mice which had been presensitized with either killed or live *M. tuberculosis* organisms; another three peptides were only weakly stimulatory. These epitopes have been immunodominant in both H-2^b and H-2^k mouse strains, indicating the genetically permissive nature of their recognition. Furthermore, both major immunodominant epitopes were found to be species specific for the *M. tuberculosis* complex and therefore potentially suitable for the early diagnosis of tuberculous infection.

INTRODUCTION

T-cell-mediated immunity against *Mycobacterium tuberculosis*, the causative agent of tuberculosis, plays a major role in the protection as well as in the pathogenesis of the disease.[1] Identification of antigenic determinants within mycobacterial proteins is therefore important for an understanding of the interactions involved in the immune response to *M. tuberculosis* and may be of potential value in the design of diagnostic reagents and subunit vaccines.

Initial studies with monoclonal antibodies and gene cloning identified the 16,000 MW antigen (Ag) (originally classified as 14,000 MW) and revealed the presence of at least one B-cell epitope highly specific for the *M. tuberculosis* complex.[2–4] Gene sequencing identified 144 amino acid residues with a molecular mass of 16,277, which possessed 30% sequence identity with proteins belonging to the α-crystallin family of low molecular weight heat-shock proteins.[5] This family includes the 18,000 MW Ag from *M. leprae* and the 22,000 MW heat-shock protein of *Chlamydomonas*.[6,7] Analysis of synthetic peptides derived from the sequence of the 16,000 MW protein with several *M. tuberculosis*-specific monoclonal antibodies (mAb) revealed the localization of two sequential epitopes, but suggested that the most prominent serological epitope (TB68) is conformational in nature.[8]

The pronounced immunogenicity of the 16,000 MW Ag is reflected by the presence of antibodies in about 70% of smear-positive and 50% of smear-negative patients with pulmonary tuberculosis.[9] However, its diagnostic value is somewhat compromised by the fact that antibody levels are also elevated in healthy subjects after occupational[10,11] or household[9] exposure to tuberculosis. This latter finding, together with the serological positivity in child tuberculosis[12] and in tuberculous meningitis,[13] indicated that the 16,000 MW Ag, when compared with other antigens, is selectively immunogenic in the early stages of subclinical infection and in primary tuberculosis. Murine T-cell proliferation *in vitro* and delayed-type hypersensitivity reactions have previously been reported only in respect of the whole recombinant 16,000 MW protein.[14]

In this paper we identified the murine T-cell stimulatory determinants of the 16,000 MW Ag using overlapping synthetic peptides spanning the complete protein sequence. Immunization of inbred mouse strains with these peptides revealed a multitude of mostly genetically permissive epitopes. However, injection of *M. tuberculosis* organisms focused the T-cell repertoire merely against two strongly immunodominant epitopes.

Abbreviations: Ag, antigen; Con A, concanavalinA; H37Ra, H37Rv, *Mycobacterium tuberculosis* strains H37Ra and H37Rv; IFA, incomplete Freund's adjuvant; LN, lymph node; mAb, monoclonal antibody; PBS, phosphate-buffered saline.

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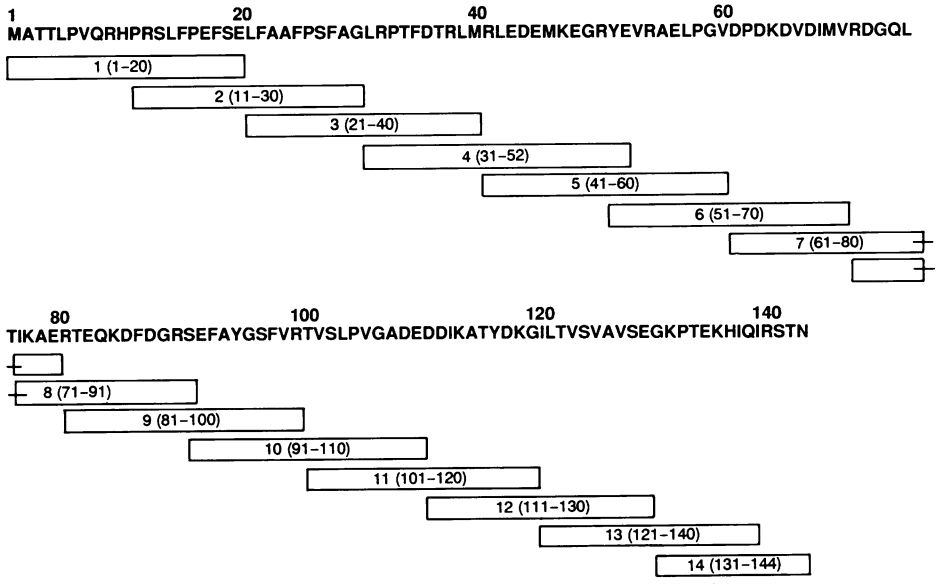

Figure 1. Synthetic peptides from the *M. tuberculosis* 16,000 MW Ag. The sequence is given by the one-letter code for amino acids. The positions of synthetic peptides, labelled 1–14, are shown within boxes.

MATERIALS AND METHODS

Mice

C57Bl/10 (H-2^b), B10.BR (H-2^k), and (B10.BR × C57BL/10) F_1 (F_1) (H-$2^{b/k}$) mice were obtained from Olac Harlem (Shaws Farm, Bicester, U.K.). In all experiments, mice were sex and age matched and used at 6–10 weeks.

Antigens

The heat-killed preparation of H37Ra was obtained from Difco Laboratories (Detroit, MI). Soluble extracts from H37Ra, *M. avium*, *M. vaccae*, *M. scrofulacium*, *M. leprae* and *M. smegmatis* were prepared as previously described.[9] *Mycobacterium tuberculosis* H37Rv was grown as a suspension culture in Middlebrook's 7H9 culture medium, harvested and stored in liquid nitrogen until required. The presence of the 16,000 MW Ag in preparations of *M. tuberculosis* was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting with the 16,000 MW-specific mAb TB68.[3] Concanavalin A (Con A) was purchased from Sigma (St Louis, MO).

Synthetic peptides

Overlapping 20mer peptides covering the complete sequence of the 16,000 MW protein were synthesized by simultaneous multiple peptide synthesis as described previously.[8,15] Briefly, Fmoc technology was employed using trialkoxy-diphenyl-methylester resin and Castro's reagent (pyBOP) for coupling. After cleavage with trifluoroacetic acid and deprotection, the peptides were purified by reverse phase high-performance liquid chromatography (HPLC) in 0·1% trifluoro acetic acid/acetonitrile followed by gel filtration through Sephadex G15 in 25% acetic acid in water. Homogeneity was confirmed by analytical reverse phase HPLC, amino acid composition and sequence by Edman degradation. The location, sequence and nomenclature of peptides are shown in Fig. 1.

Immunization procedures

Groups of three to five mice were sensitized by one of the following procedures. (1) Subcutaneous injection into the footpads with 80 μg of synthetic peptide, 50 μg of killed H37Ra organisms, 25 μg of the soluble mycobacterial extracts or phosphate-buffered saline (PBS) (control) emulsified in IFA. Popliteal lymph nodes (LN) were removed 8 days later. (2) Intraperitoneal injection of 50 μg (dry weight) of killed H37Ra organisms emulsified in IFA followed 3 weeks later by four biweekly booster injections of 25 μg of organisms suspended in PBS. Spleens were removed 10 weeks after the first injection. (3) Intraperitoneal infection with 10^6 H37Rv viable organisms. LN cells and spleen cells were harvested after 10–14 weeks.

Mouse lymphocyte proliferation assay

LN cell suspensions were prepared and used for proliferation assays as described previously.[15] Spleen cells were depleted of red blood cells by lysis with NH_4Cl and enriched for T cells by passage through nylon wool columns. When necessary, irradiated spleen cells (3000 rads) were used as a source of antigen-presenting cells (APC). Briefly, cells were suspended in RPMI-1640 medium supplemented with 5% fetal calf serum (FCS) (Gibco, Paisley, U.K.), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulphate. Triplicate cultures of 4×10^5 cells/well either alone or in the presence of 2×10^5 spleen cells were incubated in the presence of synthetic peptide in flat-bottomed 96-well plates (Nunc, Roskilde, Denmark) at 37° in an atmosphere of 5% CO_2. Cells were labelled with 37 kBq/well [^{3}H]thymidine (Amersham International, Amersham, U.K.) on the third day of culture and harvested 6–8 hr later onto glass fibre filters. The radioactive incorporation was determined by standard liquid scintillation counting. Results are expressed as means of triplicate cultures in which SD values did not exceed 15%. Preliminary analysis of peptides at 5–50 μg/ml concentration showed optimal responses at the upper dose range and therefore only results obtained at 50 μg/ml are presented.

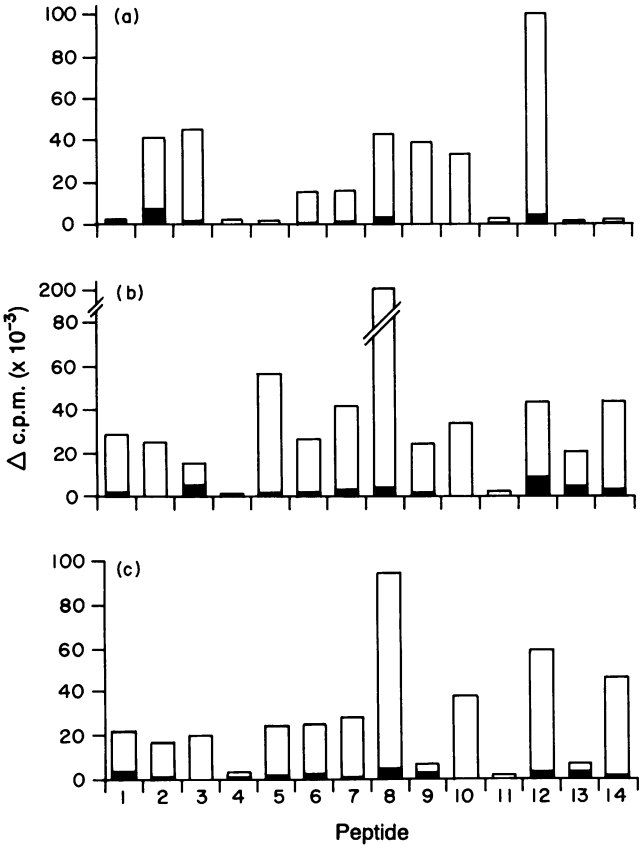

Figure 2. T-cell proliferative responses of LNC from peptide-immunized mice: (a) C57BL/10; (b) B10.BR; (c) (C57BL/10 × B10.BR)F_1. Mice were immunized into the hind footpads with either 80 μg of synthetic peptide in IFA (□) or with PBS/IFA (■). The draining LN were removed after 7 days and LN cells challenged *in vitro* with homologous peptide at a concentration of 50 μg/ml. Data are represented as mean [^{3}H]thymidine incorporation of triplicate cultures, with background values (medium alone) substracted (Δ c.p.m.).

RESULTS

Proliferative responses to peptides following homologous peptide priming

The proliferative responses of LN cells harvested 8 days after priming with each of the 14 peptides are shown in Fig. 2. In C57BL/10 mice, radioactive incorporation was at least twice, but most frequently, more than three times elevated above the PBS background values in response to peptides 2, 3, 6, 7, 8, 9, 10 and 12. B10.BR mice responded to the same peptides and also to peptides 1, 5, 13 and 14. (B10.BR × C57BL/10)F_1 mice reflected the responses in the two parent strains with two exceptions: they did not respond to peptides 9 and 13 which gave rise to proliferative responses in the C57BL/10 and B10.BR parent strains. LN cells from control mice injected with PBS/IFA did not respond significantly to any of the peptides.

To define more precisely the epitopic regions, peptide-immune LN cells were challenged *in vitro* with adjacent overlapping peptides. This analysis identified at least 11 distinct epitopes, shown for peptides 3, 6, 8 and 12 in Table 1 (data for peptides 1, 2, 5, 7, 9, 10, 13 and 14 are not shown). Interestingly, peptide 4 which failed to sensitize either strain *in vivo* was stimulatory for LN cells primed with peptide 3.

Table 1. Analysis of epitopic regions with overlapping peptides*

Priming peptide	Challenging peptide	Proliferative responses (SI)† C57BL/10	Proliferative responses (SI)† B10.BR	Epitopic region (amino acid residues)
3	2	1·1	1·1	31–40
	3	**7·8**	**3·5**	
	4	**2·4**	**2·3**	
6	5	1·0	1·0	51–70
	6	**3·3**	**3·2**	
	7	1·0	1·1	
8	7	1·1	0·9	71–91
	8	**14·5**	**15·3**	
	9	0·9	1·1	
12	11	1·1	0·9	111–130
	12	**6·5**	**21·8**	
	13	1·1	1·3	

* C57BL/10 and B10.BR mice were immunized into hind footpads with priming peptide in IFA and LN cells challenged *in vitro* with overlapping peptides at 50 μg/ml.

† Proliferative responses are expressed as stimulation indices, SI (= c.p.m. with peptide/cpm without peptide). Positive responses (SI ≥ 2) are in bold print. All proliferative responses to peptides of LN cells from control mice injected with PBS/IFA: SI ≤ 1·3.

Peptide responses following sensitization with either heat-killed or viable tubercle bacilli

Anti-peptide proliferative responses of C57BL/10 and B10.BR splenic T cells were tested after hyperimmunization with heat-killed H37Ra (see Materials and Methods). The results (Fig. 3) showed a pronounced proliferative response to peptides 3/4 and 8 and weaker responses to peptides 6 and 12 at in both strains, thus indicating their genetically permissive nature. LN cell preparations 7 days after immunization with killed bacilli produced weaker responses, but of similar peptide specificity (data not shown).

We next investigated the proliferative responses of lymphocytes prepared from spleens which had been removed 10 weeks after infection with viable H37Rv organisms. The results (Fig. 4) obtained in C57BL/10 mice showed that pronounced proliferative responses were induced only by peptides 3 and 8, while the remaining 12 peptides failed to stimulate responses above background levels. The proliferative responses to two representative non-stimulatory peptides (peptides 6 and 9) are also shown in Fig. 4. Similarly, strong selective lymphocyte responses to peptides 3 and 8 were also observed in *M. tuberculosis*-infected B10.BR and (B10.BR × C57BL/10)F_1 mice (data not shown).

Responses to peptides 3 and 8 are *M. tubcerculosis* specific

It was of interest to investigate whether the T-cell epitopes within peptides 3 and 8 were restricted to tubercle bacilli or more

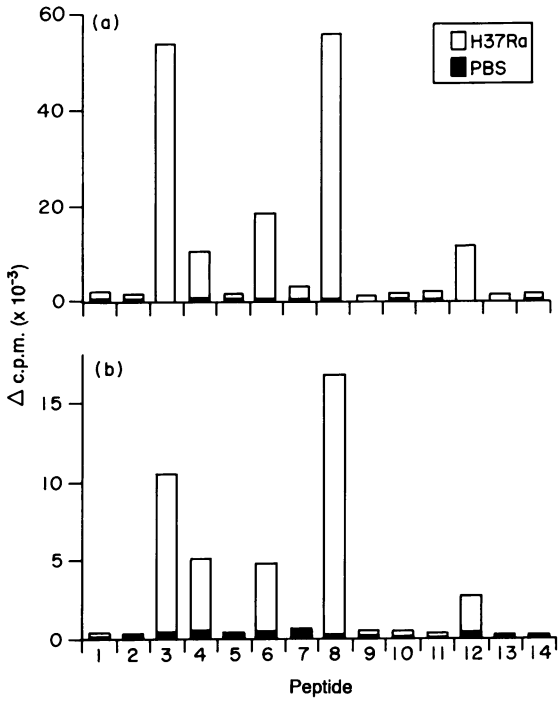

Figure 3. Anti-peptide T-cell responses after sensitization of C57BL/10 and B10.BR mice with heat-killed tubercle bacilli. Proliferative responses to peptides (1–14) from immunized mice (□) and from PBS/IFA-injected mice (■). Data are presented as Δ c.p.m. (see legend to Fig. 2). (a) Responses of nylon wool-purified spleen cells from C57BL/10 mice after immunization with heat-killed H37Ra organisms in IFA. (b) Responses of nylon wool-purified splenic T cells from B10.BR mice hyperimmunized with heat-killed H37Ra in IFA.

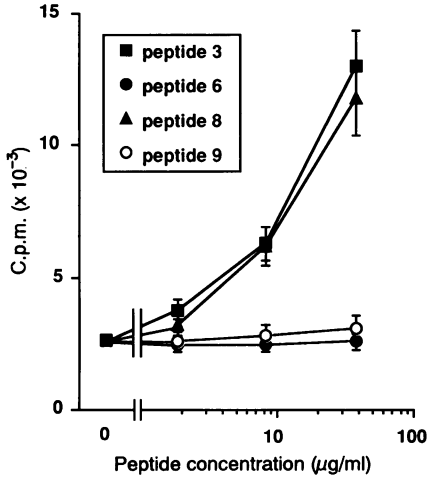

Figure 4. Anti-peptide T-cell responses after infection of C57BL/10 mice with viable tubercle bacilli. Mice were infected with live H37Rv, 10 weeks later spleen cell suspensions were prepared and incubated *in vitro* with synthetic peptides 1–14. Only results obtained with peptides 3, 6, 8 and 9 are shown. Data are presented as mean incorporation of [^{3}H]thymidine ± SD after 3 days of culture.

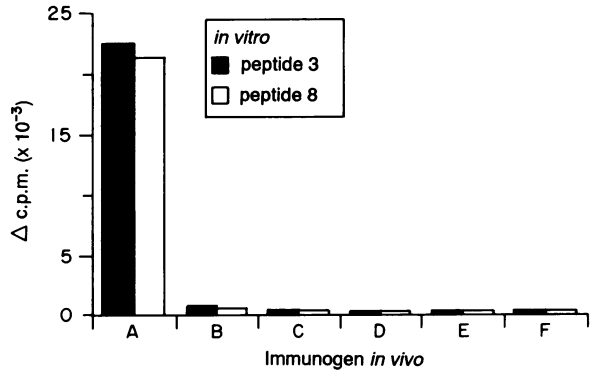

Figure 5. Species specificity of the response to peptides 3 and 8. Proliferative responses of LNC from C57BL/10 mice immunized in hind footpads with soluble extracts from different mycobacterial species: (A) H37Ra; (B) *M. smegmatis*; (C) *M. leprae*; (D) *M. vaccae*; (E) *M. avium*; (F) *M. scrofulacium*. LN cells were prepared 7 days after injection and cultured in the presence of 50 μg/ml peptide 3 (■) or peptide 8 (□) for 3 days. Results are expressed as Δ c.p.m. (see legend to Fig. 2).

widely distributed throughout the mycobacterial genus. Therefore, anti-peptide responses were determined in C57BL/10 mice following immunization with soluble extracts prepared from five non-tuberculous mycobacterial species. The results from a representative experiment (Fig. 5) showed that both peptides 3 and 8 induced proliferative responses only in LN cells from mice immunized with *M. tuberculosis* but not in LN cells from mice immunized with any of the other mycobacterial species. As a positive control, all LN cell suspensions responded to stimulation with Con A and to the corresponding homologous mycobacterial extracts (data not shown).

DISCUSSION

Priming of mice with 14 synthetic peptides spanning the whole sequence of the 16,000 MW Ag showed that the great majority of peptides, represented by 12 peptides in B10.BR and eight peptides in B10 mice induced proliferative responses when draining LN cells were stimulated *in vitro* with peptide. Considering that T-cell reactions towards adjacent peptides were revealed in only one instance (see Table 1, peptides 3 and 4), the results suggest an abundance of potential epitopes which can bind to major histocompatibility complex (MHC) class II molecules and can be recognized by the receptors of CD4 T cells. This general outcome is surprising, when compared with the relative paucity of epitopic regions within the mycobacterial 19,000 MW Ag which has been evaluated by the same experimental approach.[16]

In contrast with the priming potency of 12 peptides, a striking focusing of the T-cell repertoire was discovered following sensitization with the whole antigenic molecule. LN cells obtained from mice which had been immunized with killed tubercle bacilli produced strong responses only to peptides 3 and 8 and somewhat weaker responses to peptides 4, 6 and 12. The selection of these dominant epitopes could have been due to their high-affinity binding to MHC class II molecules. This explanation seems applicable to peptide 8 which has been the strongest stimulant in peptide-primed B10.BR and (B10.BR × C57BL/10)F_1 mice, but less so for peptide 3, which has been one of the weakest stimulants in the same mouse

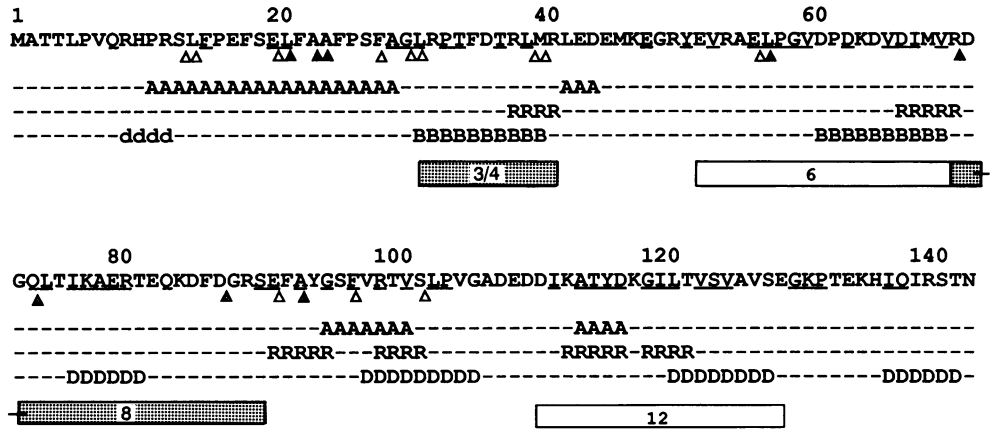

Figure 6. Localization of experimentally identified epitopes in comparison with predicted sites. The positions of four immunodominant T-cell stimulatory peptides are shown in boxes. Two of them, stimulatory in live infection are in shaded boxes. **B**, epitopes detected by mAb.[8] Epitope motifs predicted by the computer program TSITES: **A**, Amphi mid-points of blocks using window size 11; **R**, Rothbard–Taylor motif; **D**, I-Ad motif; **d**, I-Ed motif. Potential cathepsin cleavage sites[34] are shown as triangles: cathepsin B (△); cathepsin D (▲); cathepsin B and D (▲). Underlined residues are homologous with the sequence of the 18,000 MW protein of *M. leprae*.[6]

strains. In the latter case, flanking regions of peptide 3 may have been responsible for its dominant immunogenicity following bacterial priming. The described focusing of dominant epitopes of the 16,000 MW Ag appears to be similar to results in the recent study of epitope specificity of alloimmune T cells which showed that only one out of four potentially immunogenic peptides derived from the polymorphic regions of mouse MHC class II molecules has been stimulatory for T cells primed by skin allografting.[17] In both experimental systems, the results suggest that selection of immunogenic peptides from a multideterminant Ag is influenced by the mode of immunization which probably leads to distinct mechanisms of antigen processing.

It is important to note that the dominant epitopes were stimulatory in both H-2^k and H-2^b strains, thus indicating the selection of a repertoire which is focused towards genetically permissive epitopes. Preliminary results obtained in man have also revealed that peptides 3 and 8 are recognized in the context of multiple different HLA class II molecules (G. Friscia, H.-M. Vordermeier, D. P. Harris, C. Moreno and J. Ivanyi, manuscript in preparation). These findings agree with the reported permissiveness of the dominant epitopes of the 19,000 and 38,000 MW mycobacterial Ag[15,16,18] and of a variety of other Ag such as tetanus toxoid, malaria circumsporozoite protein and bovine RNase.[19–21] However, our data indicate the need to reconsider general estimates which suggest that only a minority of about 25% of randomly selected peptides can bind permissively to more than three HLA-DR haplotypes whereas the majority (~75%) bind only to one or two haplotypes.[22]

In contrast with dominant epitopes, proliferative responses to four of the eight identified cryptic epitopes were observed only in H-2^k mice. This restriction pattern corresponds to that previously reported for the antibody response to the conformational TB68 epitope.[23] It is of interest to note that (B10.BR × C57BL/10)F_1 mice showed low antibody levels, but high proliferative responsiveness to all four H-2^k-restricted peptides. In view of this genetic and functional disparity, and of the cryptic nature of these T-cell stimulatory epitopes, it has not been possible to allocate the helper function in respect of B-cell stimulation to any of these peptides. The mechanisms of this uncommon and poorly understood form of genetic control of antibody responsiveness could be elucidated perhaps by further analysis of the lymphokine secretory profile of the responding T cells.

Cryptic epitopes (i.e. immunogenic only after priming with peptides) have previously been identified and attributed to several possible mechanisms such as: (1) destruction of the epitope, either during processing of the whole antigen or of the naked peptide by proteolytic enzymes;[24] (2) interference of an adjacent or distant hindering peptide structure;[25,26] (3) preemptive competition by peptides with higher binding affinity to the MHC molecules;[26,27] or (4) limited and retarded expression of epitopes protected from proteolytic processing by binding to the surface immunoglobulin of B cells.[28] In contrast with this last explanation it was suggested that binding to surface Ig may lead to selective association with MHC class II molecules and preferential presentation to T cells.[28] This latter view is supported by the overlapping localization of immunodominant epitope 3/4 and 6 with the linear epitopes[8] recognized by mAb, represented by residues 31–40 and 61–70 (see Fig. 6).

The obtained empirical localization of epitopes was compared with the predictive analysis of the sequence, using the TSITES computer program (MedImmune Inc., Gaithersburg, MD)[29] (Fig. 5). Peptides 3/4 contained residues common to the Rothbard motif,[30] while peptide 8 contains the putative I-A^d binding motif.[31] Peptide 12 has been predicted on the basis of amphipatic α-helical structure[32] and of the Rothbard as well as I-A^d-binding motifs. It should also be noted that peptide 12 contained the highest degree of homology (11 residues) with the 18,000 MW protein of *M. leprae* and overlapped at its carboxy-terminal part with an immunodominant epitope of that Ag.[33] Using an algorithm described by Mouritsen *et al.*[34] potential cathepsin D and B cleavage sites were identified clustered in the region of amino acid residues 13–30 and 88–107 with additional sites around residues 38–40 and 57–60 (Fig. 6). This topographic association could explain the cryptic nature of epitopes within peptides 2 and 9, but not the selective lack of *in vivo* priming potency of peptide 4. The latter feature, observed also in other mycobacterial Ag[15,35] could be due to susceptibility to extracellular enzymes or to a role of flanking sequences that enhance the affinity of peptide binding to MHC molecules.

The fact that we did not observe significant proliferative responses to peptides 6 and 12 after infection with live organisms could be due to enhanced activities of either proteolytic enzymes or stress protein chaperones in macrophages harbouring the intracellular tuberculous infection. This could result in lower peptide concentrations available at the cell surface of APC for presentation to T cells which would favour the recognition of the strongest epitopes. It is also tempting to speculate that the T-cell populations primed by live infection to peptides 3 and 8 are of different specificity from those sensitized with killed bacilli (e.g. in terms of their cytokine production profiles).

Irrespective of these considerations, concerning the host–parasite relationship, the results are pertinent for the development of new diagnostic reagents. In particular, the immunodominant epitopes within peptides 3 and 8 which are genetically permissive and species specific for *M. tuberculosis* could be useful for the detection of T-cell responses of the infected host by either lymphocyte responsiveness *in vitro* or by skin testing. This potential application is supported by previous evidence of overlapping T-cell immunodominance in the murine and human species[36] and is also being confirmed by initial evaluation of human responses to the 16,000 MW antigenic peptides (G. Friscia, H.-M. Vordermeier, D. P. Harris, C. Moreno and J. Ivanyi, manuscript in preparation).

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