

On the diversity and heterogeneity of H-2^d-restricted determinants and T cell epitopes from the major bee venom allergen

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Abstract

One of the main limitations of using synthetic peptides for immunotherapy in allergic patients is the difficulty to delineate the immunodominant T cell epitopes which are necessarily dependent on HLA molecules. We have thus addressed the question of the role of MHC II molecules in immunodominant epitopes selection in the particular case of the major bee venom allergen (API m1). To exhaustively and easily explore it, we used BALB/c mice whose H-2 haplotype is associated with high IgE and IgG responses to API m1. By means of extensive sets of synthetic peptides, we investigated the specificity of polyclonal T cells and monoclonal hybridomas from mice immunized with API m1 and delineated four immunodominant regions, restricted to either the I-E^d or the I-A^d molecule. All the peptides were also tested for their capacity to bind to immunopurified MHC II molecules. Eight determinants of high affinity were identified. They clustered into three distinct regions and were largely overlapping. They included all the immunodominant epitopes, but half of them were not capable of stimulating T cells. Strikingly, interacting surfaces with either the TCR or MHC II molecule greatly differed from one determinant to another. In one case, we observed that flanking regions exerted a particular action on T cell stimulation which prevented the fine epitope localization. Our results underline the diversity and complexity of MHC II-restricted determinants and T cell epitopes from the major bee venom allergen, even in a single haplotype. These data also participate in the development of alternative approaches to conventional immunotherapy.

Introduction

Allergen-specific CD4⁺ T lymphocytes, isolated from peripheral blood of atopic patients or identified at the site of allergic contact, actively participate in disease progression (1). They generally produce high levels of IL-4 and IL-5 but little IFN- γ , and hence allow B lymphocytes to differentiate into IgE-secreting plasmocytes. Interestingly, successful specific immunotherapy with grass pollen and insect allergens correlates with alteration of the cytokine profile, skewed to a T_H1 pattern (2,3), or with decline of T cell proliferation (4,5). These observations suggest that allergen-specific T cells are the cellular targets of immunotherapy and therefore offer a possible rational basis to design new molecules for desensitization, hopefully devoid of IgE-mediated anaphylactic side

effects (6,7). Ideally, these molecules should possess all the determinants that are efficiently recognized by the allergen-specific CD4⁺ T lymphocytes, i.e. the immunodominant epitopes. Their design, however, is seriously limited by the difficulty to precisely define MHC II-restricted epitopes and hence requires a better understanding of what governs the immunodominance of T cell epitopes.

Immunodominant T cell epitopes are carried by peptides, produced by the antigen degradation in antigen-presenting cells (APC) and presented by MHC II molecules to CD4⁺ T cells. In the mouse, various experimental systems have demonstrated that immunodominance results from a complex and dynamic combination of factors stemming from antigen

processing (8,9), MHC II specificity (10) and TCR repertoire (8,11). Antigens are trapped by APC and are degraded into flexible fragments of various lengths by concomitant actions of acidic environment, disulfide bond reduction and proteolytic cleavage (12). Peptide rescue by MHC II molecules occurs in different compartments of the endocytic route but depends on the peptide binding strength and the pH conditions at the meeting point (13). It is facilitated by the capacity of MHC II molecules to bind long antigenic fragments (9). It is, however, disturbed by the editing function of HLA-DM/H2-M (14,15) and the regulated expression of HLA-DO/H2-O (16). Finally, T cell recruitment also requires a sufficient number of T cell precursors able to recognize the presented peptides (8,11). CD8⁺ T cell responsiveness to class I-restricted epitopes is equally regulated by various, albeit different, factors including cellular machinery, TAP and MHC specificity (17). However, due to accurate and simple methods of epitope prediction available for MHC class I molecules (18) but not for MHC II molecules, several studies have quantified the relative contribution of each factor. They have revealed the predominant influence played by affinity to the MHC class I molecule (19–21). MHC II molecules also exert a major control on T cell response (22,23) and reactivity (10,11). However, their precise role on immunodominance remains to be identified, and requires an extensive and comparative study on T cell epitopes and MHC II-restricted determinants, which can be easily monitored in mice.

We have thus investigated, in BALB/c mice, the MHC II molecules influence on immunodominant epitopes selection in the special case of bee venom phospholipase A2 (API m1). BALB/c mice share with H-2^d and H-2^k mice the ability to produce good levels of specific IgE and IgG upon immunization with API m1 (24). In humans, API m1 is recognized as the major bee venom allergen since most prick-test-positive patients displayed API m1-specific IgE (25). Some API m1 T cell lines and clones have also been isolated. They displayed various peptide specificities and HLA restrictions (26–28), illustrating their large diversity. In this paper, we delineated multiple H-2^d-restricted immunodominant epitopes, using polyclonal T cells and monoclonal hybridomas. Affinity for immunopurified H-2^d molecules was then compared to that from non-T cell-stimulating peptides. We revealed a nested distribution of MHC II-restricted determinants and T cell epitopes of various size.

Methods

Antigens

API m1 (Sigma, St Quentin Fallavier, France) was purified on a C₄ Vydac column using an acetonitrile/0.1% trifluoroacetic acid gradient to eliminate melittin. Reduced API m1 was obtained after treatment at room temperature in darkness with an excess of DTT in 0.1 M Tris-HCl, 6 M guanidine chloride, pH 8, buffer. It was purified as described for native API m1. Two sets of 18 and 13 amino acid-long peptides were defined on the basis of the amino acid sequence of API m1, deduced from its cDNA (29). These peptides and the peptide hemagglutinin (HA) 306–318 (PKYVKQNTLKLAT) were synthesized on an Advanced ChemTech 357 MPS synthesizer (Advanced

Chemtech Europe, Brussels, Belgium) according to the Fmoc strategy. Fmoc-protected amino acids, Rink Amide Resin and HBTU were purchased from Novabiochem (France Biochem, Meudon, France). Side chain protecting groups were chosen as followed: tert-butyloxycarbonyl for lysine and tryptophan, tert-butyl for serine, threonine and tyrosine, 2,2,5,7,8-pentamethyl chroman-6-sulfonyl for arginine, and trityl for asparagine, cysteine, glutamine and histidine. Biotinylated HA 306–318 (bHA) was obtained by coupling biotinyl-6-aminocaproic acid (Fluka Chimie, St Quentin Fallavier, France) on the N-terminus of HA 306–318, following the same protocol as for Fmoc amino acids. Peptides were deprotected, and cleaved from the resin by treatment with 95% trifluoroacetic acid, 2.5% triisopropylsilane and 2.5% distilled water. They were purified to homogeneity by reversed-phase HPLC on a C₁₈ Vydac column using an acetonitrile/0.1% trifluoroacetic acid gradient. Their quality was assessed by electrospray mass spectroscopy. The peptide myoglobin (MYO) 106–118 (FISEAIHVLHSR) and its biotinylated form (bMYO) were purchased from Neosystem (Strasbourg, France).

Polyclonal T cell activation assay

Ten-week-old female BALB/c mice (Charles River, Cléon, France) were immunized s.c. with 110 µg of native API m1 emulsified in complete Freund's adjuvant (CFA). A second injection was done in incomplete Freund's adjuvant (IFA) 28 days later. Eleven days after this second injection, spleen cells were harvested and resuspended in RPMI 1640 (Biological industries, ATGC, Noisy le Grand, France) supplemented with 1% autologous normal mouse serum, 4 mM glutamine, 2 mM sodium pyruvate, 10 mM HEPES, 500 µg/ml gentamycin and 50 µM 2-mercaptoethanol. Spleen cells (5×10⁵ cells/well) were plated in triplicate into 96-well microtiter plates (Nunc, Roskilde, Denmark) with appropriate concentrations of API m1 or API m1 peptides. After 4 days of culture at 37°C, cells were harvested following the addition of [³H]thymidine (1 µCi/well, 5 Ci/mmol; Amersham, Little Chalfont, UK) during the final 16 h of culture. Incorporated thymidine was determined by scintillation counting in a β counter (Wallac 1410; Egg, Evry, France). In some experiments, T cell activation was measured by incubating 10⁶ splenocytes during 24 h at 37°C at an optimal concentration of peptide and by submitting the supernatants to CTLL assay. Briefly, after extensive washes, 10⁴ IL-2-dependent CTLL cells, suspended in RPMI 1640 supplemented with 5% FCS, 2 mM glutamine, 10 mM HEPES, 500 µg/ml gentamycin and 50 µM 2-mercaptoethanol, were added to warmed culture supernatants and incubated for 24 h. [³H]Thymidine (1 µCi/well, 5 Ci/mmol; Amersham) was added and the cells were harvested 6 h later. Incorporated thymidine was determined as previously described.

API m1-specific T cell hybridomas

API m1 T cell hybridomas were generated as described previously (30). Briefly, female BALB/c mice between 6 and 10 weeks old were immunized at the base of the tail with 50 µg of native API m1 emulsified in CFA. Two other immunizations (50 and 100 µg) were repeated in IFA emulsion at 2 weeks intervals. Fifteen days after the last injection, spleen cells were harvested and cultured for 4 days in the presence of 1 µM API m1, in RPMI 1640 medium supplemented with

10 mM HEPES, 4 mM glutamine, 2 mM sodium pyruvate, 500 µg/ml gentamycin, 50 µM 2-mercaptoethanol and 1% fresh autologous normal mouse serum. Cultured cells and BWα⁻ cells (a kind gift from Dr. J.-G. Guillet, Hopital Cochin, Paris, France) were fused at a 1:1 ratio with polyethylene glycol 1500 (Boehringer Mannheim, Meylan, France) and then plated in 96-well microtiter plates with 2.5×10^5 spleen cells. After HAT selection, growing hybridomas were screened for API m1 specificity. They were cultured with 1 µM API m1 and 5×10^5 splenocytes from BALB/c mice for 24 h. Supernatants were submitted to CTLL assay. Positive T cells were cloned by limiting dilutions and maintained in DMEM (Biological Industries) supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 500 µg/ml gentamycin, 1% non-essential amino acids, 10 mM HEPES and 50 µM 2-mercaptoethanol (DMEM medium). In standard conditions, activation assays were performed with 5×10^4 T cell hybridomas and 5×10^5 splenocytes as APC. For experiments using fibroblasts, 5×10^4 I-E^d- or I-A^d-transfected fibroblasts (a kind gift from Dr J.-G. Guillet, Hopital Cochin, Paris) were plated into 96-well plates 1 day before the assay.

Purification of MHC II molecules

The A20.11.1 cell line was a kind gift from Professor H. M. McConnell (Stanford University, CA). 14.4.4S and MKD6 hybridomas were obtained from ATCC (Rockville, MD). These cells were maintained in DMEM medium. mAb were purified from cell culture supernatants on a Protein A-Sepharose column as described by the manufacturer (Pharmacia, St Quentin en Yvelines, France). 14.4.4S and MKD6 antibodies were coupled to respectively Protein A-Sepharose CL 4B gel and CNBr-activated Sepharose 4B gel (Pharmacia). A20.11.1 cells were lysed on ice at 10^8 cells/ml in 150 mM NaCl, 10 mM Tris-HCl, pH 8.3, buffer containing 1% Nonidet P-40, 10 mg/l aprotinin, 5 mM EDTA and 10 µM PMSF. After centrifugation at 100 000 *g* for 1 h, the supernatant was applied to Sepharose 4B and Protein A-Sepharose 4B (Pharmacia) columns, and then to the 14.4.4S- and the MKD6-affinity columns. I-E^d and I-A^d were eluted respectively from the 14.4.4S- and the MKD6-affinity columns with 1.1 mM *n*-dodecyl β-D-maltoside (DM), 500 mM NaCl and 500 mM Na₂CO₃, pH 11.5, buffer. Fractions were immediately neutralized to pH 7 with 2 M Tris-HCl, pH 6.8, buffer and extensively dialysed against 1 mM DM, 150 mM NaCl, 10 mM phosphate, pH 7, buffer.

MHC II-peptide binding assays

The MHC II molecules I-E^d and I-A^d were diluted in 10 mM phosphate, 150 mM NaCl, 1 mM DM, 10 mM citrate, 0.003% thimerosal buffer at pH 5 (I-E^d) or pH 6 (I-A^d) with bHA or bMYO respectively and with serial dilutions of competitor peptides. The samples were incubated in 96-well polypropylene plates (Nunc) at 37°C for 24 or 72 h and under reductive conditions (2 mM DTT) for I-A^d. After neutralization with 0.45 M Tris-HCl, pH 7.5, 0.003% thimerosal, 0.3% BSA, 1 mM DM buffer, samples were applied to 96-well Maxisorp ELISA plates (Nunc) previously coated with 10 µg/ml 14.4.4S (for I-E^d) or MKD6 (for I-A^d) mAb and saturated with 100 mM Tris-HCl, pH 7.5, 0.3% BSA, 0.003% thimerosal buffer. Samples were allowed to bind to the antibody-coated plates

for 2 h at room temperature. Bound biotinylated peptide was detected by incubating streptavidin-alkaline phosphatase conjugate (Amersham), and after washing, by adding 4-methylumbelliferyl phosphate substrate (Sigma). Emitted fluorescence was measured at 450 nm upon excitation at 365 nm on a Fluorolite 1000 fluorimeter (Dynex, Issy les moulineaux, France). Maximal binding was determined by incubating the biotinylated peptide with the MHC II molecule in the absence of competitor. Binding specificity was assessed by adding an excess of the reference peptide. Background was low and did not significantly differ from that obtained by incubating the biotinylated peptide without MHC II molecules. Data were expressed as the inverse of the peptide concentration which prevented binding of 50% of the labeled peptide (IC₅₀). Average and SE values were deduced from at least three independent experiments. The HA 306–318 and MYO 106–118 peptides exhibited an IC₅₀ equal to 10^{-7} M for I-E^d and I-A^d respectively.

Results

T cell response to API m1 in BALB/c mice involves three immunodominant regions

To delineate the immunodominant T cell epitopes from API m1 in BALB/c mice, we used 18 amino acid-long peptides which encompassed the whole API m1 sequence (29) and overlapped by 14 residues. All possible 15 amino-acid-long peptides present in the API m1 sequence were thus included within the 30 peptides we synthesized. They allowed us to cover as close as possible the potential T cell epitopes, since 15 amino acids was the most frequently found length of naturally processed peptides (31). BALB/c mice were immunized with API m1 and their spleen cells re-stimulated *in vitro* with synthetic peptides. T cell proliferation was observed for native API m1 and for seven peptides, i.e. P21–38, P25–42, P73–90, P77–94, P81–98, P109–126, P113–130. They defined three non-overlapping regions (Fig. 1). IL-2 secretion was also used to evaluate T cell activation and led to identical conclusions (data not shown). Since BALB/c mice express two different MHC II molecules, we determined the restriction elements supporting peptide-specific T cell re-stimulation by means of I-E^d- or I-A^d-specific mAb (Fig. 2). Although an irrelevant antibody did not suppress T cell activation in a significant manner, stimulation by the P77–94, P81–98, P109–126 and P113–130 peptides was mainly diminished by the I-E^d-specific antibody as compared to the I-A^d-specific one. It was, however, the opposite for the P21–38 and P25–42 peptides. We inferred from these data that T cell stimulation related to P77–94, P81–98, P109–126 and P113–130 peptides was restricted to I-E^d, while the P21–38 and P25–42 peptides re-stimulated I-A^d-restricted T cells.

In order to identify the T cell response against API m1, API m1-specific monoclonal T cell hybridomas were isolated and characterized for peptide and MHC II specificities. As described above, restriction was investigated by antibody inhibition experiments (data not shown) but also using I-E^d- or I-A^d-transfected fibroblasts. Data obtained from both methods converged to identical conclusions. Fibroblasts are known to weakly present native proteins as compared to

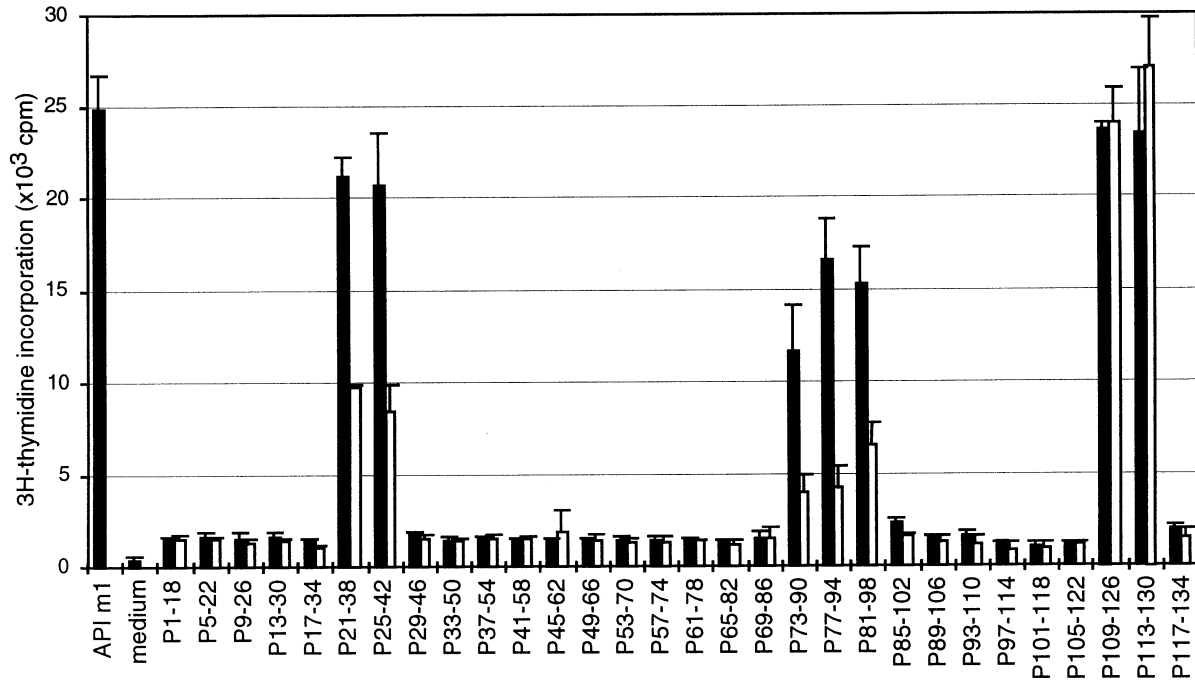


Fig. 1. Delineation of API m1 immunodominant T cell epitopes. Spleen cells from BALB/c mice immunized with native API m1 were cultured in the presence of API m1 (0.08 μ M) or 18mer peptides at different concentrations (solid bars, 2 μ M; open bars, 0.4 μ M). Proliferation was determined by [^{3} H]thymidine incorporation. Error bars represent SD calculated from triplicate cultures. Data presented are from one of two independent experiments.

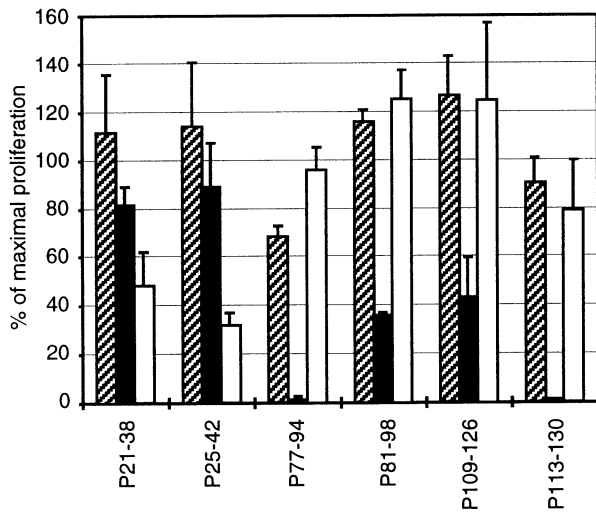


Fig. 2. Restriction of API m1 immunodominant T cell epitopes. Splenocytes from BALB/c mice immunized with native API m1 were cultured with optimal concentration of peptides (2 μ M for P21-38, P25-42 and P113-130, and 10 μ M for P77-94, P81-98 and P109-126) and 5 μ g/ml antibodies (striped bars, L243 = anti HLA-DR; solid bars, 14.4.4S = anti I-E^d; open bars, MKD6 = anti I-A^d). IL-2 secretion was determined by CTLL assay. Background was estimated with medium alone and varied from 202 to 775 c.p.m. Maximal proliferation, determined without any antibodies, depended on the stimulating peptides, and was 8988, 7157, 7488, 15792, 13131 and 5725 c.p.m. for the P21-38, P25-42, P77-94, P81-98, P109-126 and P113-130 peptides respectively.

denatured antigens (32). We thus performed these experiments using native and reduced API m1. As illustrated in Fig. 3, only two types of hybridomas were found. Four hybridomas, including 6A4.4 clone, were stimulated by the P21-38 and P25-42 peptides only (Fig. 3A). No other peptides were stimulating. These hybridomas were stimulated by reduced and native API m1 presented by I-A^d-transfected fibroblasts, although I-E^d-transfected cells were totally inefficient (Fig. 3B). They were therefore unambiguously I-A^d-restricted. On the contrary, 5G5.7 T cells as five other hybridomas were restricted to I-E^d molecules (Fig. 3D). I-E^d-transfected fibroblasts provoked T cell stimulation with both reduced and native API m1, the I-A^d-transfected cells being inactive. These T cell hybridomas were specific to the P73-90, P77-94 and P81-98 peptides (Fig. 3C). No other hybridomas were isolated, and in particular no T cells specific to the P109-126 and P113-130 peptides were found. Thus, for at least two of the immunodominant regions, monoclonal T cells confirmed both peptide specificity and MHC II restriction. We concluded that three T cell immunodominant regions existed upon immunization of BALB/c mice with API m1: I-A^d/21-42, I-E^d/73-98 and I-E^d/109-130.

Immunodominant peptides are included among the best binders of I-A^d and I-E^d molecules

The 30 overlapping 18mer peptides which spanned the entire API m1 sequence were then tested for their capacity to bind I-E^d and I-A^d immunopurified class II molecules. These assays were based on a competitive ELISA, and allowed us to easily evaluate the binding strength of a large number of peptides

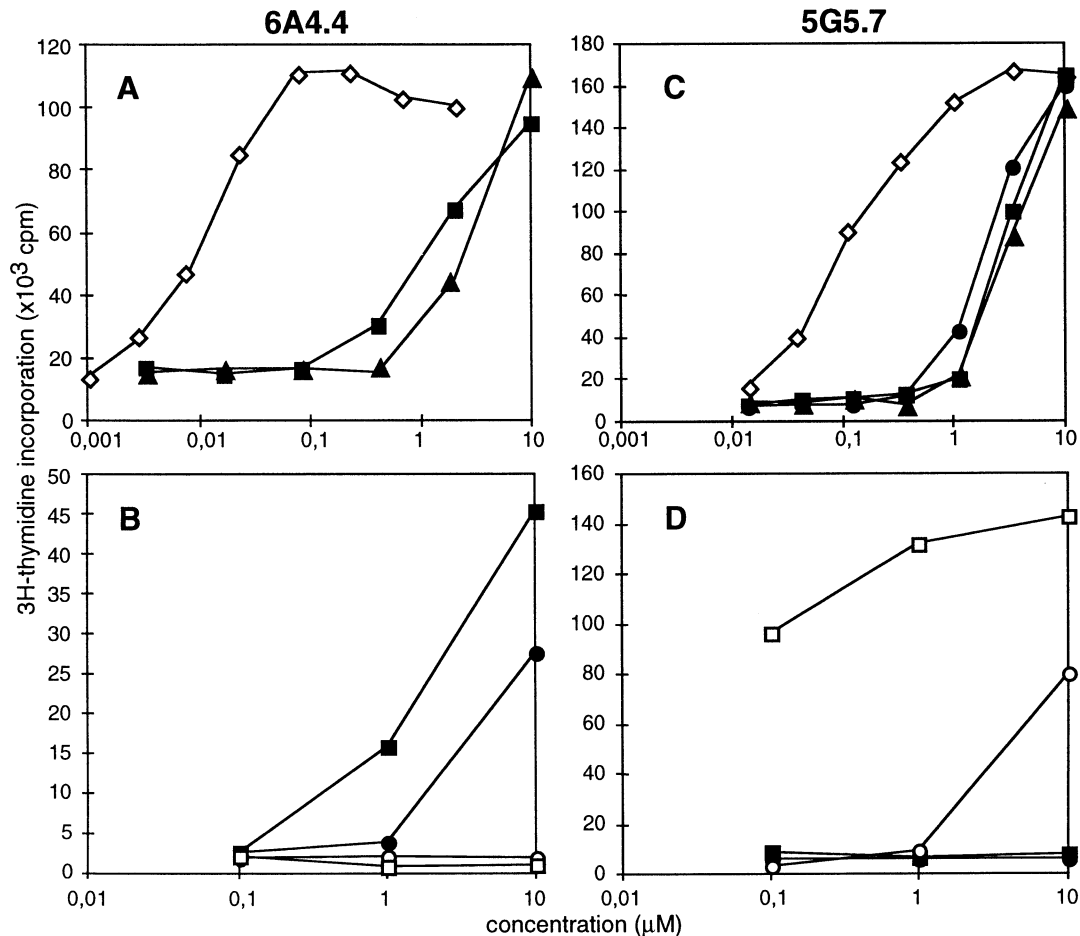


Fig. 3. Specificity and restriction of API m1-specific T cell hybridomas. (A and C) Each hybridoma was cultured with fresh splenocytes and different concentrations of API m1(◇) or 18mer peptides (for 6A4.4: ■, P21-38; ▲, P25-42; for 5G5.7: ■ P73-90, ▲ P77-94; ●, P81-98). IL-2 secretion was determined by CTLL assay. Background estimated with medium alone was 14337 and 11708 c.p.m. for 6A4.4 and 5G5.7 respectively. (B and D) Each hybridoma was cultured with fibroblasts expressing on their membrane either I-E^d (open symbols) or I-A^d (close symbols), and native (circles) or reduced (squares) API m1 at different concentrations. IL-2 secretion was determined by CTLL assay. Background estimated with medium alone was 2288 and 5834 c.p.m. for 6A4.4 and 5G5.7 respectively. Variations in duplicate and triplicate cultures did not exceed 15%. Data presented are from one of at least two independent experiments.

in a sensitive and reproducible manner. Results are reported Fig. 4 as inverse of IC_{50} to facilitate comparison with those from T cell stimulation assays. Nine of the 30 peptides tested with the I-E^d-specific assay (i.e. 30% of the peptides) yielded $IC_{50} < 10^{-5}$ M and defined three non-overlapping regions. The remaining peptides were inactive. Seven peptides (P73-90, P77-94, P81-98, P109-126, P113-130 and P117-134) exhibited IC_{50} close to 10^{-7} M while two other peptides had IC_{50} between 10^{-5} and 10^{-6} M. Interestingly, all I-E^d-restricted immunodominant peptides belonged to the seven best binder peptides. Nevertheless, P85-102 and P117-134 peptides did not stimulate T cells although they bound to I-E^d as well as dominant peptides. I-A^d-specific binding assays revealed five peptides with IC_{50} spreading from 10^{-5} to 10^{-6} M (P21-38, P25-42, P73-90, P77-94, P81-98). They represented one-sixth (16%) of the 30 peptides tested. They clustered in two different regions which largely overlapped I-E^d binding ones since four peptides (P21-38, P73-90, P77-94, P81-98) bound efficiently both I-A^d and I-E^d molecules (Fig. 4). As described

above for the I-E^d molecule, the two I-A^d-restricted peptides (P21-38 and P25-42) that stimulated T cells also exhibited best binding potencies to the I-A^d molecule. As a result, MHC II-binding assays revealed 14 different MHC II-peptide complexes corresponding to five different binding combinations (I-E^d/17-38, I-A^d/21-42, I-E^d/73-102, I-A^d/73-98 and I-E^d/109-134). They contained all the immunodominant epitopes identified above.

Fine delineation of MHC II-binding regions reveals a mixed population of determinants

In order to precise the contact areas of API m1 determinants with the I-E^d and I-A^d molecules, a second set of 13 amino-acid-long peptides was synthesized. They encompassed the five binding regions defined above and overlapped by 12 amino acids. The length of 13 residues was chosen as a compromise. It was the minimal length of naturally processed peptides (31,33) and was expected to be short enough to discriminate two binding contact areas in a single 18mer

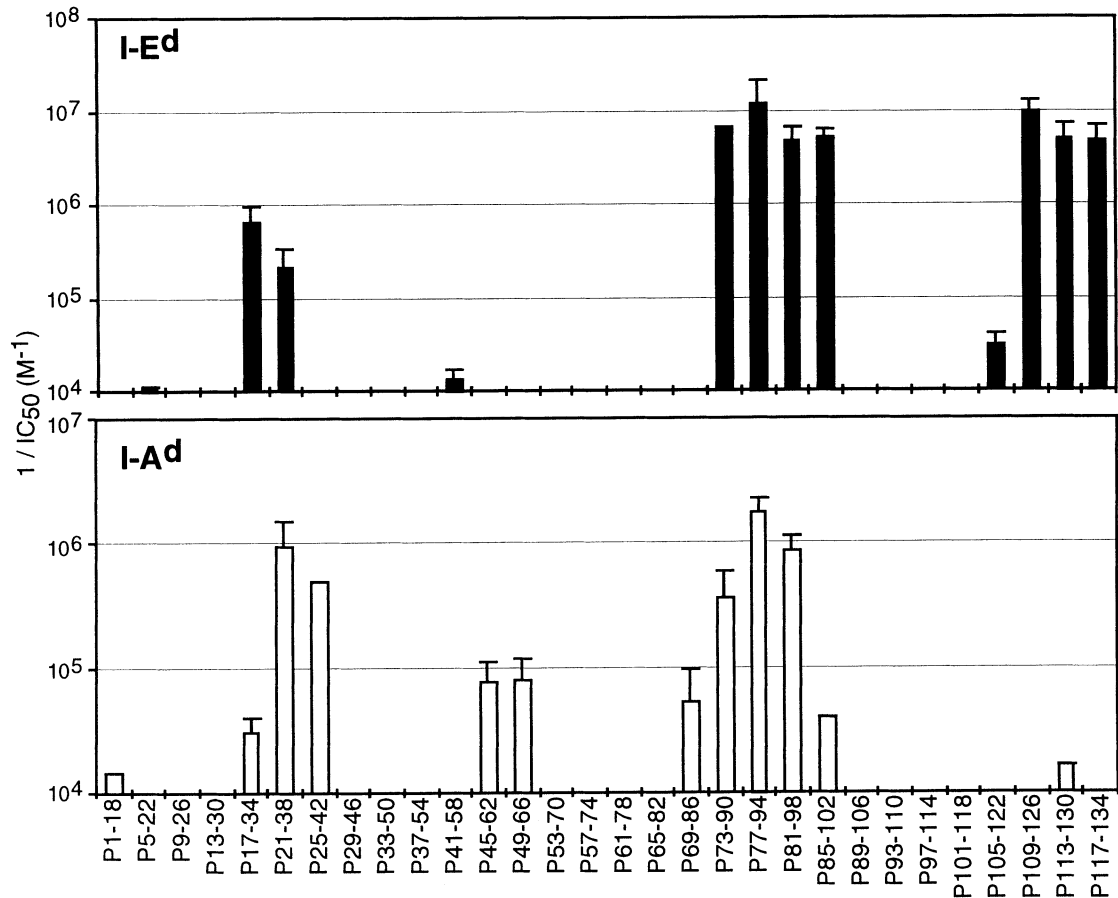


Fig. 4. Binding capacities of 18mer peptides to purified I-E^d and I-A^d molecules. Biotinylated bHA (3×10^{-8} M) and bMYO (10^{-7} M) peptides were incubated for 24 h with I-E^d and I-A^d molecules respectively, and various dilutions of API m1 peptides. Binding activity was quantified as described in Methods and resulted from at least three independent experiments. Lack of histogram indicates that the peptide displayed no inhibitory activity at the maximum concentration of 10^{-4} M. Reference peptides (HA 306–318 and MYO 106–118) were included in each binding assay with their respective MHC II molecule. Their IC₅₀ was 10^{-7} M and variation did not exceed a factor of 3.

peptide. In the 17–42 region of API m1, three 13mer peptides (P20–32, P21–33 and P22–34) efficiently bound to I-E^d (Fig. 5A). They were as efficient as the corresponding 18mer peptides, suggesting that the interacting surface could be <13 amino acids long. In contrast, the P25–37 peptide bound significantly less to I-A^d than the P21–38 peptide did (Fig. 5D). Thus, optimal interaction of this determinant required a peptide length >13 amino acids. A more complex situation was observed in the central part of API m1. The 73–102 fragment was shown to encompass two distinct I-E^d binding determinants (Fig. 5B). One comprised the P77–89, P78–90, P79–91, P80–92, P81–93 and P82–94 peptides. The N-terminal part of the determinant was thus located between position 77 and 82, while the C-terminal end was placed between position 89 and 94. The minimal length of this determinant was estimated to range from 8 to 13 amino acids. The second I-E^d-binding region was defined by the P85–97 peptide. It bound to I-E^d as well as the P81–98 and P85–102 18mer peptides did, and was separated from the first determinant by two inactive 13mer peptides (P83–95 and P84–96 peptides). In this API m1 central zone, I-A^d-binding assays also revealed two separated determinants. Seven consecutive peptides (P75–

87, P76–88, P77–89, P78–90, P79–91, P80–92 and P81–93) had similar IC₅₀ which did not significantly differ from that of the P81–98 18mer peptide. They encompassed one or several binding surfaces which could be from 7 to 13 amino acids long. Only one inactive peptide separated them from the second interacting region which was formed by the P83–95 and P84–96 peptides. These two peptides exhibited similar efficiency to bind to I-A^d as compared to the P81–98 18mer peptide. Finally, the C-terminal part of API m1 was the site of two I-E^d binding regions (Fig. 5C). The first one comprised the P112–124, P113–125, P114–126 and P115–127 peptides which were as efficient binders as the corresponding P109–126 and P113–130 18mer peptides. The P121–133 and P122–134 peptides defined the second one, and corresponded to the active P117–134 18mer peptide. We thus identified at least eight different determinants which interacted with MHC II molecules. Five and three of them were restricted to I-E^d and I-A^d respectively (see summary Table 1 and Fig. 6).

T cell reactivity of the 13mer peptides reveals an additional immunodominant peptide

The same set of 13mer peptides was used to investigate the fine specificity of API m1-specific polyclonal T cell population

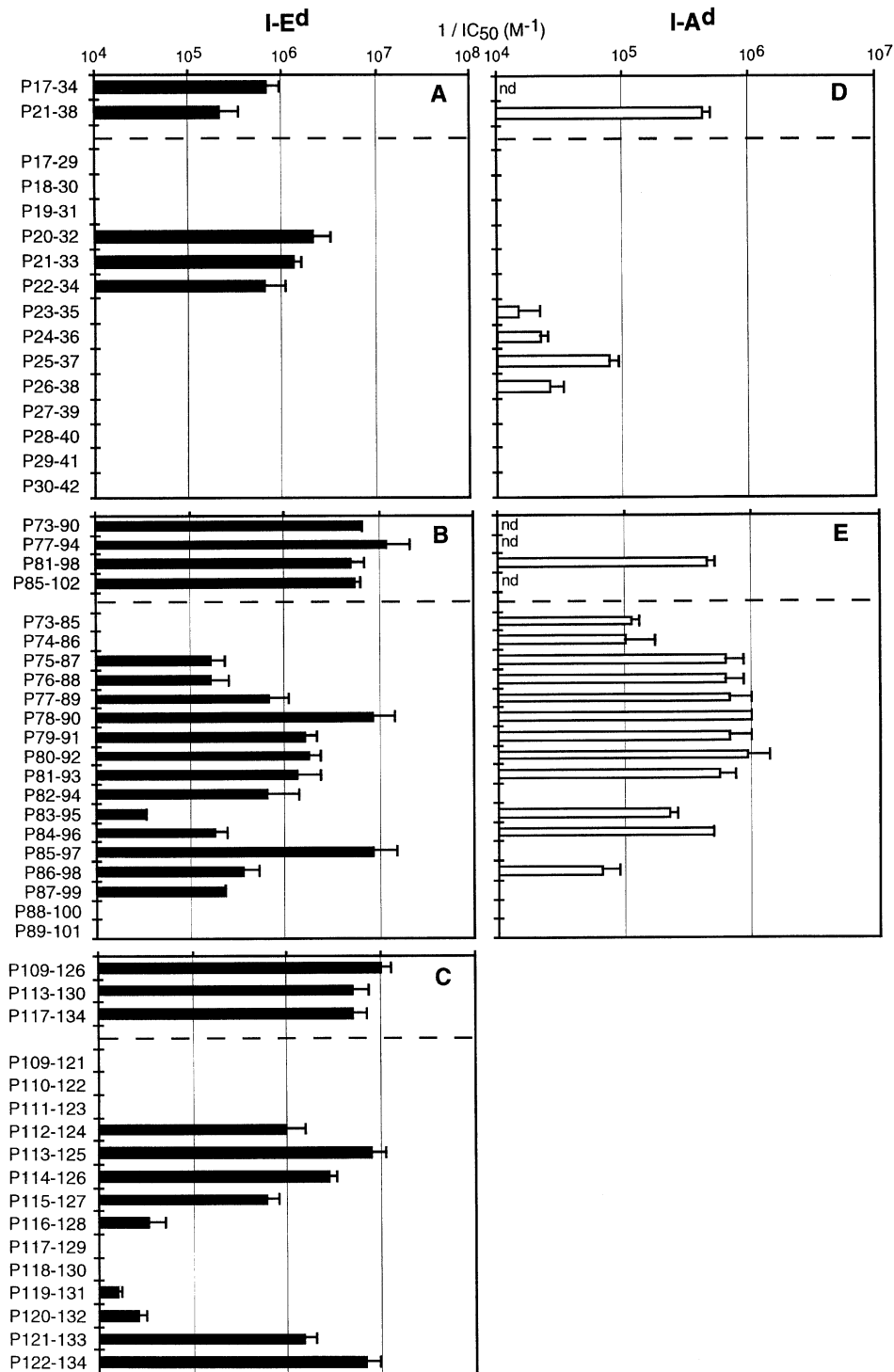


Fig. 5. Binding capacities of 13mer peptides to purified I-E^d and I-A^d molecules. Biotinylated bHA (3×10^{-8} M) and bMYO (2×10^{-7} M) peptides were incubated respectively for 24 h with I-E^d and 72 h with I-A^d, and several concentrations of API m1 peptides. Binding activity was measured as described in Methods and in the legend of Fig. 4. Comparison with 18mer peptides which were added to these experiments is done at the top of each graph. nd, not determined in these experiments

and monoclonal T cell hybridomas. In the N-terminal part of API m1, only the P25–37 peptide was T cell stimulating (Fig. 7A). As compared to the P21–38 peptide, it was dramatic-

ally less active to stimulate T cells even at the maximal dose of 30 μ M. The same low potency was observed with two 21–42-specific hybridomas as illustrated with 6A4.4 T cells

Table 1. Summary of the T cell response to API m1 in BALB/c mice: characteristics of MHC II-binding determinants and T cell epitopes

H-2 ^d molecule	MHC II-binding determinants			T cell epitopes			
	N-terminal	C-terminal	Length	Dominant	N-terminal	C-terminal	Length
I-E ^d	20–22	32–34	11–13	no	–	–	–
I-E ^d	77–82	89–94	8–13	yes	73–81	90–98	≥14
I-E ^d	85	97	13	no	–	–	–
I-E ^d	112–115	124–127	10–13	yes	112–115	124–127	10–13
I-E ^d	121–122	133–134	12–13	no	–	–	–
I-A ^d	21–25	38–42	≥14	yes	21–25	38–42	≥14
I-A ^d	75–81	87–93	7–13	no	–	–	–
I-A ^d	83–84	95–96	12–13	yes ^a	≤83	≥95	≥13

To localize the MHC II-restricted determinants and the T cell epitopes, we have considered the sets of 18mer and 13mer peptides displaying the maximal binding or stimulating activity. For each set, we have distinguished the sequence shared by all the peptides (common part) from the ones surrounding it (surrounding parts). Since a peptide can bind to a MHC II molecule by several ways, N- and C-termini of the interacting surface are located between the limits of the common and surrounding parts. Its length varies between the size of the common region and that of the shortest active peptide (see also Fig. 7).

^aRestriction of the peptide P83–95 has been deduced from its capacity to bind exclusively. I-A^d molecule.

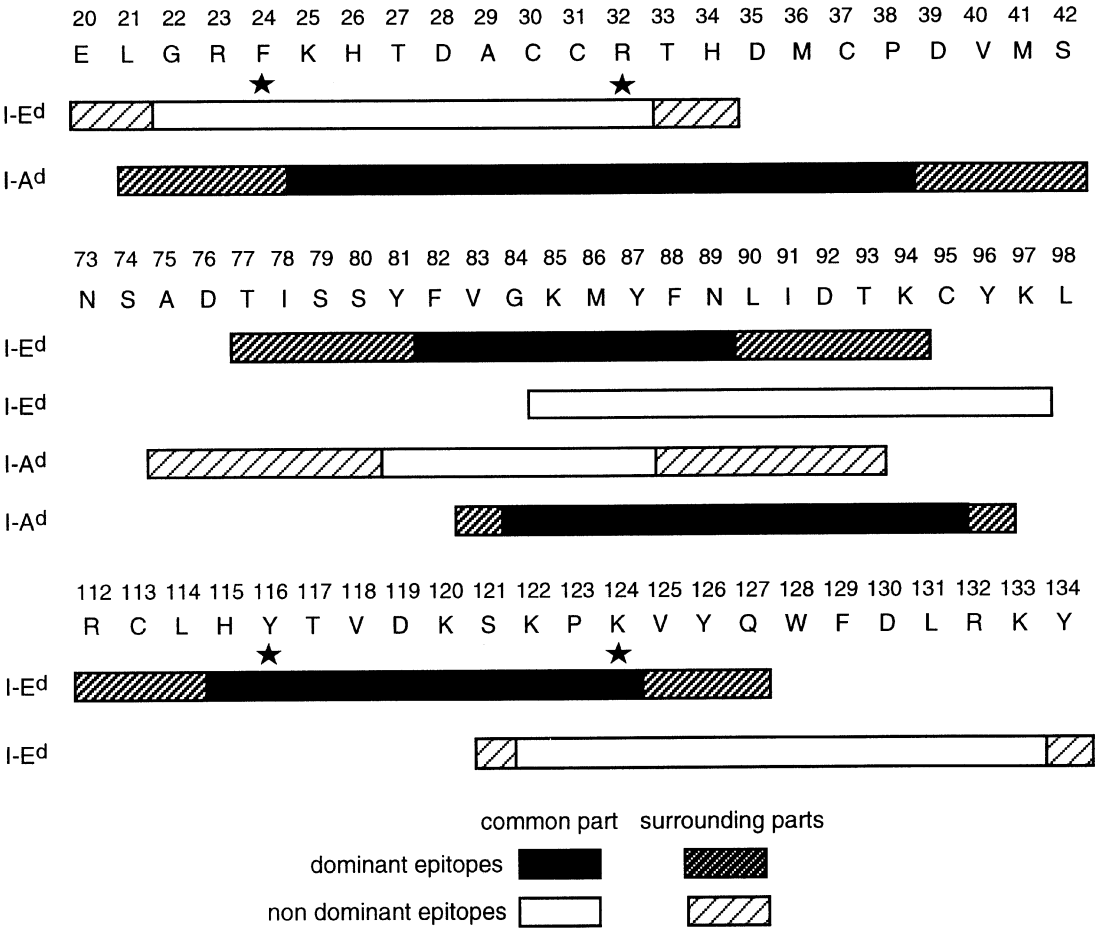


Fig. 6. Diversity and heterogeneity of I-E^d and I-A^d binding determinants from API m1. The identified I-E^d- and I-A^d-binding determinants were reported on the sequence of API m1 (29). The limits of their common and surrounding parts were deduced from the binding experiments as described in the legend of Table 1. Only the terms dominant epitopes and non-dominant epitopes refer to T cell stimulation results. An asterisk indicates the residues involved in the I-E^d-binding motif deduced from natural peptide elution and sequencing (51).

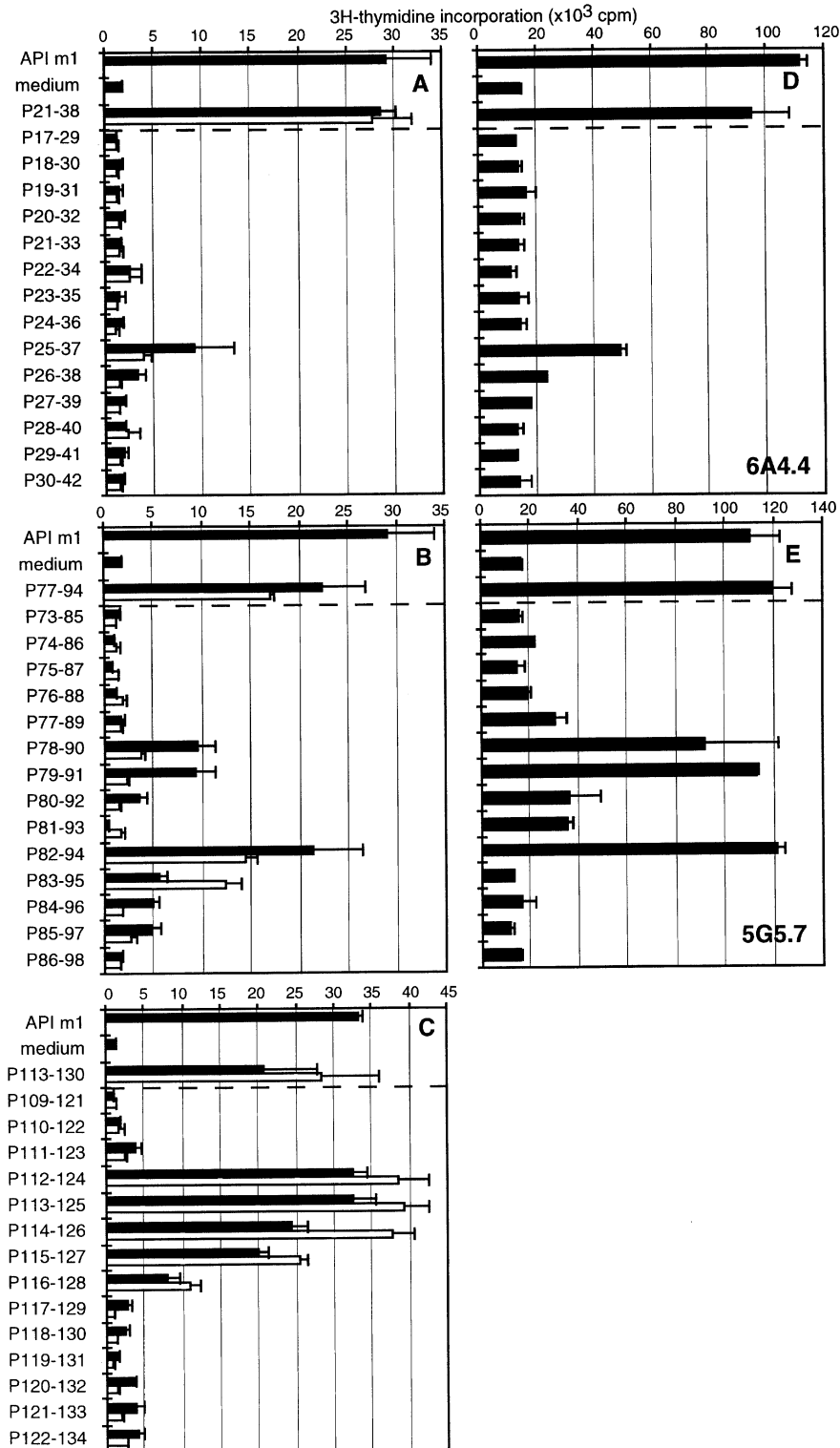


Fig. 7. Specific T cell activation by 13mer peptides. Spleen cells (A–C) from API m1-primed mice were cultured with API m1 (0.4 μM) or 18mer and 13mer peptides (solid bars, 30 μM ; open bars, 6 μM). Proliferation was determined by [^3H]thymidine incorporation. Hybridomas 6A4.4 (D) and 5G5.7 (E) were cultured with fresh splenocytes and API m1 (0.2 μM) or API m1 peptides (18mer: 10 μM ; 13mer: 30 μM). IL-2 secretion was determined by CTLL assay. Activities of API m1, negative control and 18mer peptides are shown at the top of each graph. Error bars represent SD calculated from triplicate (A–C) or duplicate (D and E) cultures. Data presented are from one of two independent experiments.

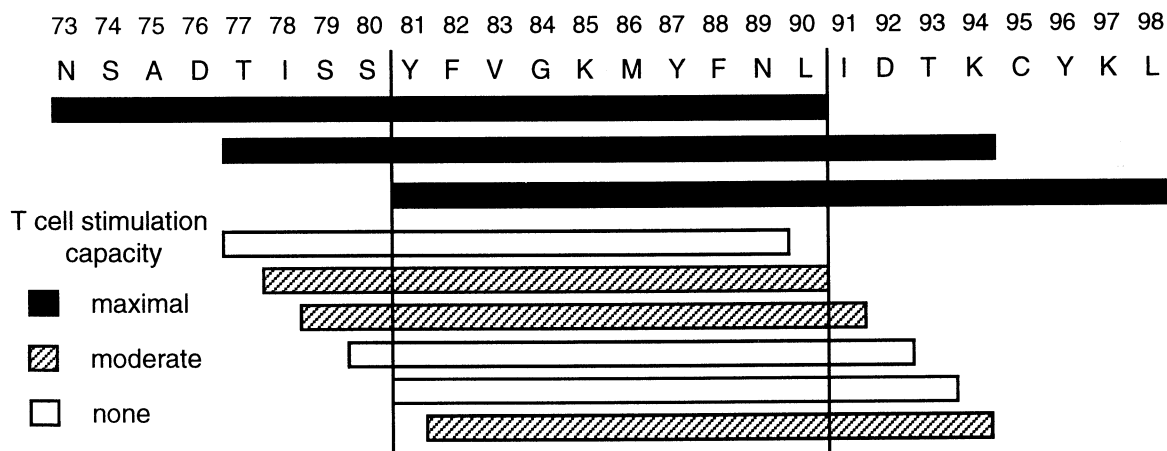


Fig. 8. Influence of the I-E^d/77-94 epitope flanking regions on T cell recognition. Capacities of the I-E^d/77-94-related peptides to activate monoclonal or polyclonal API m1-specific T cells are presented by a shading code. Maximal activity refers to the 18mer peptides which are stimulating at concentrations <10 μ M. Moderate indicates that stimulation occurs at higher concentration and mainly at 30 μ M. None refers to the lack of stimulation. Vertical bars encompass the common part of the three 18mer peptides.

(Fig. 7D). This was related to the decreased binding strength of the P25-37 peptide to I-A^d. The corresponding epitope should thus extend to >13 residues. In the central part of API m1, four non-consecutive peptides (P78-90, P79-91, P82-94 and P83-95) stimulated at a high concentration the polyclonal T cells (Fig. 7B) and apparently represented two different epitopes. Four T cell hybridomas specific to this region were activated by only three peptides, i.e. P78-90, P79-91 and P82-94 (Fig. 7E). Since cloned hybridomas recognize a single T cell epitope, the P78-90, P79-91 and P82-94 peptides belonged to the same I-E^d-restricted epitope. As they were dramatically less active than the corresponding 18mer peptides (see Fig. 3C), each of them did not entirely contain the T cell epitope which was centered on the I-E^d/77-94 binding region. This T cell stimulation pattern was all the more striking as the 18mer P73-90, P77-94 and P81-98 peptides were equally active and shared a common part of 10 residues (Fig. 8). Moreover, this I-E^d/77-94 epitope did not account for T cell reactivity of the P83-95 peptide that did not bind to I-E^d but to I-A^d. The P83-95 peptide may therefore participate in another immunodominant epitope contained in the P81-98 peptide and restricted to I-A^d. It should, however, produce less specific T cells than the corresponding I-E^d-restricted epitope, since antibody inhibition experiments did not reveal it (Fig. 2). In the C-terminal part of API m1, P112-124, P113-125, P114-126 and P115-127 peptides stimulated polyclonal T cells as well as the P113-130 18mer peptide, suggesting that the size of this epitope could be <13 amino acids (Fig. 7C). These peptides fitted exactly the corresponding region which bound to I-E^d. Finally, no T cell stimulation was observed for the P20-32, P75-87, P85-97 or P122-134 peptides which efficiently bound to an MHC II molecule. It confirmed the lack of T cell reactivity for I-E^d/17-34, I-A^d/75-93, I-E^d/85-102 and I-E^d/117-134 determinants (Fig. 1).

Discussion

Recent advances regarding specific immunotherapy have underscored the potential to use T cell epitopes containing

molecules to desensitize patients (34). However, identified T cell epitopes from allergens are highly diverse and hence of limited clinical use (26-28). We have therefore investigated the relationships between T cell epitopes and MHC II molecules by thoroughly analyzing the BALB/c mice T cell response to the major bee venom allergen (API m1). We have identified eight determinants in the API m1 sequence which interact efficiently with MHC II molecules and which can be grouped into three distinct regions: 20-42, 75-97 and 112-134. Among them, three to four immunodominant epitopes (I-E^d/77-98, I-E^d/112-127, I-A^d/21-42 and the presumed I-A^d/83-95 epitope) account for the T cell response to API m1 in BALB/c mice. Three major points have emerged from our results (see summary Table 1 and Fig. 6).

First, we have described in detail the relation between affinity of peptides for MHC II molecules and their immunodominance. In particular, we have observed that all dominant epitopes are present in best binder peptides in agreement with previous data (8,35). Peptide affinity is generally related to its capacity to form stable complexes with MHC II molecules (36-38). Such a property may therefore enhance peptide resistance to HLA-DM/H-2M proofreading and may facilitate peptide presentation to T cells (15). As a result, immunodominant epitope location in native antigen sequence is primarily dictated by MHC II specificity as suggested by immunization experiments in congenic mice (39,40). In particular, we have not found any relationship between epitope immunodominance and API m1 secondary structures. However, we have observed that half of the binding determinants do not lead to T cell stimulation, indicating that, although necessary, a good affinity is not sufficient for a peptide to be immunodominant (8). Interestingly, these determinants systematically overlap a dominant T cell epitope, restricted either to the same MHC II molecule or to the other one (Fig. 6). The most striking pattern has been found in the central part of API m1 which harbors at least four contact areas with I-E^d or I-A^d, two of them being capable of activating T cells. Nevertheless, the following T cell epitopes, I-E^d/77-94, I-E^d/112-127 and I-A^d/83-95, do not

exhibit better IC₅₀ than their non-stimulating neighbours, respectively I-E^d/85–97, I-E^d/121–134 and I-A^d/75–93. Immunodominant epitopes could not therefore prevent the presentation of their non-dominant counterparts by competition for the same MHC II molecule. On the other hand, we cannot exclude that the lack of stimulation by the I-E^d/20–34 determinant results from competition with the I-A^d/21–42 dominant T cell epitope. Such a competition between epitopes restricted to different MHC molecules but carried by a same peptide (41) does not probably take place in API m1 central part because of the nested distribution of determinants (Fig. 6). It would require that I-E^d/77–94 competes with I-A^d/75–93 but not with I-A^d/83–95 or that the latter interferes with the loading of I-E^d/85–97 but not of I-E^d/77–94. For the same reason, a preferential cleavage in this central region that would destroy the non-dominant epitopes but preserve the dominant ones is also unlikely. We thus suggest that other factors such as T cell repertoire (8,11) account for the lack of T cell stimulation from some determinants of API m1 in BALB/c mice. Therefore, MHC II molecules appear to play a similar role in immunodominance as do MHC I molecules (19–21). Clearly, peptide dominance is primarily but not entirely dictated by MHC molecules.

Second, we have underlined that difficulty to delineate MHC II-binding determinants and T cell epitopes is associated with frequent overlaps and size variations in their interacting areas. I-A and I-E molecules share a similar structure with HLA-DR molecules (42–44). Bound peptides vary in size from ~13 to 25 amino acids (31,33,45) and are free to extend out both ends of the binding site. Nevertheless, a well-ordered region from position P-2 to P10 or P11 as numbered by Stern *et al.* (42) is observed in all crystallographic structures and suggests that 12–13 amino acids are in contact with the MHC II molecule. We therefore expected to readily delineate interacting regions by an exhaustive scanning with 13 amino-acid-long peptides. For seven of the eight defined binding determinants, at least one 13mer peptide is as active as the corresponding 18mer peptide. However, this is not the case for the I-A^d/21–42 determinant, indicating that its interacting surface is >13 amino acids. In sharp contrast, the determinant I-A^d/75–93 is covered by seven 13mer active peptides and hence may extend on only 7 amino acids. A partial filling of I-A^d pockets could be enough to produce optimal interaction strength as shown by a structure recently published (44). Another non-exclusive possibility is that several overlapping binding determinants which use different anchor residues are hidden in these sequences (46) and form a mixed population of MHC–peptide complexes. Sharp differences between T cell epitopes have been also encountered. For the I-A^d/21–42 epitope, no 13mer peptide stimulates specific T cells as well as 18mer peptides, whereas optimal stimulation occurs with four different short peptides for the I-E^d/112–127 determinant. For the I-E^d/77–94 epitope, both polyclonal and monoclonal T cells are stimulated by three consecutive long peptides, i.e. P73–90, P77–94 and P81–98 (see also Fig. 8). The 81–90 common region is thus expected to suffice for optimal T cell stimulation. Nevertheless, the P80–92 and P81–93 peptides encompass it but fail to activate T cells while stimulation is partially restored for P78–90, P79–91 and P82–94 peptides. Maximal activation is reached only when the presented pep-

tide contains either a sufficient part of the 73–80 or the 91–98 surrounding regions, or both. In other words, the T cell stimulation decrease provoked by shortening the stimulating peptide at one side is compensated by elongating it at the other one. It is intrinsically not possible to delineate precisely this epitope. We suggest that these different patterns of T cell stimulation are caused by the ragged extremities of naturally presented peptides (31,33,45) and by the complex contribution of flanking regions to T cell stimulation (47).

Finally, our results also suggest several consequences regarding the allergic response in human beings and the choice of peptide sequences for specific immunotherapy. H-2^d and H-2^k haplotypes are both associated with high IgE and IgG responses to API m1 (24). Such an association resembles those existing in humans between HLA molecules and allergic disease (34,48,49). Comparing our results with those obtained in H-2^k mice (50) shows that several immunodominant peptides are involved in the T cell response and that these peptides vary from one associated haplotype to another. HLA polymorphism is currently recognized to contribute to T cell epitope diversity (34). Our data also show that diversity exists even in a single haplotype and can be easily underestimated. Finally, frequent overlaps and size variability of CD4⁺ epitopes makes their fine delineation difficult. Hence, this favors the use of long fragments which encompass the overlapping T cell epitopes (28) as an alternative to actual immunotherapy.

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Abbreviations

APC	antigen-presenting cell
API m1	major bee venom allergen
CFA	complete Freund's adjuvant
DM	<i>n</i> -dodecyl β-D-maltoside
HA	hemagglutinin
IFA	incomplete Freund's adjuvant
MYO	myoglobin

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