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H-2-associated effects of flanking residues on the recognition of a permissive mycobacterial T-cell epitope

E. ROMÁN, D. P. HARRIS, S. JURCEVIC, J. IVANYI & C. MORENO *MRC Tuberculosis and Related Infections Unit, Clinical Sciences Centre, Royal Postgraduate Medical School, Hammersmith Hospital, London, UK*

SUMMARY

Previously we have identified an immunodominant, eight-residue, epitope core sequence (TAAGNVNI) from the 19 000 MW protein of *Mycobacterium tuberculosis*, which is recognized in the context of multiple H-2 I-A molecules. In this study, the role of residues flanking this T-cell epitope core was examined, using a series of 20 mer analogue peptides in which the native flanking residues were progressively replaced with L-alanine. Analogue peptides were tested for their capacity to stimulate a $CD4^+$ 19 000 MW protein-specific T-cell line, revealing that all but one N-terminal flanking residue could be replaced collectively by alanine without significant loss of stimulatory activity. However, clear H-2-associated differences in the requirement for flanking residues were demonstrated with peptide-specific T-cell hybridomas. In particular, $H\text{-}2^d$-derived hybridomas were much more stringent in their requirement for flanking residues than were $H\text{-}2^b$ hybridomas. All polyalanine-substituted peptides bound $I\text{-}A^b$ molecules, with affinities similar to the native unsubstituted peptide. In contrast, significantly reduced binding to $I\text{-}A^d$ was observed with several analogue peptides, although without a clear relationship to the degree of substitution. Furthermore, in $H\text{-}2^b$ mice, neither immunogenicity nor cross-reactivity with the native peptide showed a clear inverse relationship with respect to the degree of alanine substitution. The results presented in this paper indicate that flanking residues can influence T-cell specificity and that these effects may be controlled by major histocompatibility complex (MHC) haplotype.

INTRODUCTION

The $\alpha\beta$ T-cell receptor (TCR), expressed on $CD4^+$ T lymphocytes, interacts with peptide–major histocompatibility complex (MHC) complexes, formed following proteolytic degradation of antigen (Ag).[1] Most of the allelic variability that distinguishes different MHC class II molecules is located within the polymorphic peptide-binding groove, which is open-ended, accommodating antigeneic fragments ranging in size from ~15 to 30 amino acids in length.[2,3] Analysis of the T-cell determinant structure has revealed that epitopes are typically composed of a six- to nine-residue long epitope 'core', contained within a variable number of so-called flanking residues.[4–6] While results from amino acid substitution experiments have shown that residues involved in MHC binding and TCR interaction are usually located within the epitope core, less is known about the effects of flanking residues on T-cell recognition.

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Abbreviations: Ag, antigen; APC, antigen-presenting cell; IC50, inhibitor concentration 50%; IFA, incomplete Freund's adjuvant; LNC, lymph node cell; MHC, major histocompatibility complex; p, peptide; poly(Ala), polyalanine; TCR, T-cell receptor.

Correspondence: Dr C. Moreno, MRC Tuberculosis and Related Infections Unit, Clinical Sciences Centre, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0NN, UK.

Studies of the T-cell response to variant gp120-derived peptides from different strains of human immunodeficiency virus (HIV), each containing a conserved epitope core sequence, showed that the flanking residues play a critical role in defining T-cell specificity.[7] Single amino acid substitutions in flanking regions outside the minimal MHC binding epitope were also reported to influence dramatically T-cell recognition of hen egg lysozyme-derived peptides.[8] The insertion of epitopes from heat-shock protein (hsp) 65 and the pre-S2 region of herpes simplex virus (HSV) into different sites within bacterial recipient proteins was shown to influence their recognition.[9,10] In these studies, it was suggested that failure to recognize the inserted epitope may have been due to 'inappropriate' processing of the epitope due to the nature of the local flanking residues. It has also been reported that flanking sequences influence the presentation of endogenously synthesized peptides recognized by cytotoxic T cells.[11] Thus flanking residues may influence epitopes recognized in the context of both class I and class II molecules and, clearly, these results have important implications for vaccines designed on the basis of introducing foreign epitopes into carrier proteins.

For this study we chose a well-defined, immunodominant epitope of the 19 000 MW protein of *Mycobacterium tuberculosis* (residues 61–80), to evaluate whether the specificity of amino acid residues that flank the epitope core affects T-cell specificity, MHC binding and immunogenicity. The epitope

core within peptide (p)61–80 is localized between residues 69–76 (TAAGNVNI) and is recognized permissively in the context of multiple class II I-A alleles.[5] Examination of the proliferative response of p61–80-specific T-cell hybridomas to singly substituted analogue peptides revealed that most residues within the epitope core were critically required for T-cell stimulation.[5] In contrast, no data are so far available regarding the contribution of flanking residues to the immunogeneic properties of this genetically permissive epitope.

In the present report we examined the effect of amino acid substitutions on peptide immunogenicity and MHC binding, using a set of analogue peptides where peptide length had been kept constant at 20 residues, while residues flanking the epitope core were progressively replaced with L-alanine. Our results indicate clear differences in the requirement for flanking residues among different H-2 haplotypes.

MATERIALS AND METHODS

Synthetic peptides

Peptides were made in a fully automatic MilliGen/Bioresearch 9050 Pepsynthesiser (Millipore, Watford, UK) using standard methods for F_{moc}–amino acid pentafluorophenyl esters on a PepSyn KB resin and according to conditions recommended by the manufacturer for the synthesis and cleavage of peptides, which were amidated at the carboxy-end of the molecule. Efficiency of the coupling cycles was monitored by ionic dye equilibrium distribution[12] and was maintained at > 98% for each residue. The procedures used for purification and characterization of synthetic peptides have been described previously.[5] The amino acid sequences of the series of polyalanine [poly(Ala)]-substituted analogue peptides used in this study are shown in Fig. 1.

Immunization procedure and preparation of lymph node cells

This procedure has been described in detail elsewhere.[5,13] Briefly, mice were immunized subcutaneously in the hind footpads with 30 nmol synthetic peptide or phosphate-buffered saline (PBS) emulsified in incomplete Freund's adjuvant (IFA), and the draining popliteal lymph nodes removed 8 days later. Single lymph node cell (LNC) suspensions were prepared in complete RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS Gibco, Paisley, UK), and added (4×10^5 cells/well) in triplicate to 96-well, flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) containing various concentrations (0–25 μM) of Ag. After 3 days incubation at 37° in 5% CO_2, cells were pulsed for 6 hr with 37 kBq/well [^{3}H]thymidine (Amersham International, Amersham, UK) and radioactive incorporation determined by liquid scintillation counting.

Proliferation assays with T-cell lines

T-cell lines were assayed for Ag-specific proliferative responses at the end of a resting phase, as described previously.[14] Cells were added (2×10^4 cells/well) to plates containing Ag (0–25 μM) and irradiated syngeneic antigen-presenting cells (APC) (3×10^5 cells/well). After 3 days incubation, microcultures were labelled with [^{3}H]thymidine and harvested as indicated previously.

T-cell hybridomas

T-cell hybridomas specific for p61–80 were established by a standard fusion protocol using PEG 1500 (Boehringer, Mannheim, Germany) in conjunction with the TCR$\alpha\beta^-$ fusion partner BW5147, as described previously.[5] Hybridoma cells were washed extensively with culture medium and incubated ($2–5 \times 10^4$ cells/well) in the presence of irradiated syngeneic spleen cells (3×10^5 cells/well) and Ag. After 24 hr culture, supernatants (100 μl/well) were removed, frozen for at least 2 hr and the IL-2 content subsequently assayed with the HT-2 indicator cell line.

Peptide-binding assay

Peptide binding to I-A^b and I-A^d molecules was tested using either purified class II MHC molecules[15] or cells expressing appropriate H-2 molecules. For binding to I-A^b and I-A^d expressed on cells, I-A^d-expressing A20 B-lymphoma cells and A20-I-A^b transfectants[16] were washed, resuspended in serum-free RPMI medium and added to 96-well microtitre plates (10^5 cells/well). A biotinylated, I-A^b-binding reference peptide (p350–369 from the 38 000 MW protein of *M. tuberculosis*)[4] or I-A^d reference peptide [ovalbumin (OVA) 323–336][17] was added to the cells at 100 μM final concentration. Unlabelled competitor peptides were added simultaneously at different

Amino acid sequence

Epitope core

Peptide	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
p1	V	T	G	S	V	V	C	T	T	A	A	G	N	V	N	I	A	I	G	G
p2	A	T	G	S	V	V	C	T	T	A	A	G	N	V	N	I	A	I	G	A
p3	A	A	G	S	V	V	C	T	T	A	A	G	N	V	N	I	A	I	A	A
p4	A	A	A	S	V	V	C	T	T	A	A	G	N	V	N	I	A	A	A	A
p5	A	A	A	A	V	V	C	T	T	A	A	G	N	V	N	I	A	A	A	A
p6	A	A	A	A	A	V	C	T	T	A	A	G	N	V	N	I	A	A	A	A
p7	A	A	A	A	A	A	C	T	T	A	A	G	N	V	N	I	A	A	A	A
p8	A	A	A	A	A	A	A	T	T	A	A	G	N	V	N	I	A	A	A	A
p9	A	A	A	A	A	A	A	A	T	A	A	G	N	V	N	I	A	A	A	A
p10	A	A	A	A	A	A	A	A	A	A	A	G	N	V	N	I	A	A	A	A
p11	A	A	A	A	A	A	A	A	A	L	A	A	N	A	N	I	A	A	A	A

Figure 1. Amino acid sequences of poly(Ala)-substituted peptides used in this study. Residues corresponding to the wild-type p1 (residues 61–80) are underlined and the boxed residues (69–76) indicate the position of the T-cell epitope core.[5]

final concentrations ranging from 50 to 1000 μM. After overnight incubation, cells were lysed using PBS/1% Nonidet P-40 (NP-40) and lysates cleared of debris by centrifugation for 5 min at 1000 *g*. Lysates were then transferred to 96-well microtitre plates, precoated (1 μg/well) with anti-I-A^b antibody (AF6-120.1.2) or anti-I-A^d antibody (MD-K6). After 2 hr incubation at 4°, the plates were thoroughly washed and the amount of biotinylated peptide bound to I-A^b or I-A^d captured on appropriate plates was determined colorimetrically using streptavidin–peroxidase (Sigma, St Louis, MO). Typically, absorbance in the presence of the biotinylated reference peptides without added competitor peptide was 0·2–0·3, while the background absorbance was 0·01–0·05. When peptide-binding was tested in parallel using purified molecules or corresponding cells, similar results were obtained.

RESULTS

T-cell stimulatory capacity of poly(Ala)-substituted peptides

To evaluate the role of residues flanking the immunodominant p61–80 epitope core, a series of 20 mer, poly(Ala)-substituted analogue peptides was synthesized (Fig. 1). The results of a representative experiment, demonstrating the capacity of analogue peptides to stimulate an H-2^b-derived T-cell line specific for the 19 000 MW protein, are shown in Fig. 2. With only minor variations, p2–8 stimulated T-cell proliferative responses as effectively as the wild-type p1. The amount of peptide required to induce 50% maximum c.p.m. ranged from 0·01 μM (p8) to 0·1 μM (p2). In contrast, p9, containing the complete epitope core intact (residues 69–76) but flanked by alanine residues, was ~100 times less potent than the control p1. The remaining peptides (p10, p11) were not efficiently recognized and elicited negligible responses. These results suggest that most, but not all, residues flanking this epitope core can be replaced with alanine without significant loss of stimulatory capacity.

We have previously observed differences in both length requirements and critical residues for p61–80 (p1) recognition in different H-2 haplotypes.[5] We therefore analysed the recognition of poly(Ala)-substituted peptides with a panel of p61–80-specific T-cell hybridomas derived from H-2^b and H-2^d mice. The proliferative response of each hybridoma was analysed with analogue peptides titrated over a 4-log range of concentrations. For the purposes of evaluation however, the results of these experiments are presented in a summarized form as relative stimulatory capacity (Fig. 3). For hybridomas derived from C57BL/10 (H-2^b) mice, responses were maintained as far as p8 (clones 7.6, 7.29) or p10 (clone 7.37). In contrast, a very early and sharp decline in the stimulatory capacity of analogue peptides was observed with all three clones (7.13, 7.31 and 7.24) derived from H-2^d mice. Thus analysis of the recognition of analogue peptides by T-cell hybridomas revealed clear differences in the requirement for flanking residues between different haplotypes and also, although to a lesser extent, among individual clones derived from the same haplotype.

Binding of poly(Ala)-substituted peptides to I-A^b and I-A^d

The same set of analogue peptides (p1–11) was used to test whether substitution of the natural flanking residues with L-alanine affected binding to I-A^b and I-A^d molecules. None of the substitutions significantly affected binding to the I-A^b molecule (Fig. 4a). Thus even p11, with all flanking residues replaced by alanine as well as four substitutions within the epitope core, bound I-A^b with a similar affinity to the wild-type p1. In contrast, binding of several analogue peptides to the I-A^d molecule was either diminished (p6, p7) or practically abolished (p8) (Fig. 4b). Therefore substitution of V65 (p6) and V66 (p7) led to a *c.* threefold decrease in binding, while additional substitution of C67 (p8) resulted in a *c.* 10-fold decrease in binding. Unexpectedly, the substitution of Thr at positions 68 and 69 (p10) was accompanied by a complete recovery of I-A^d binding.

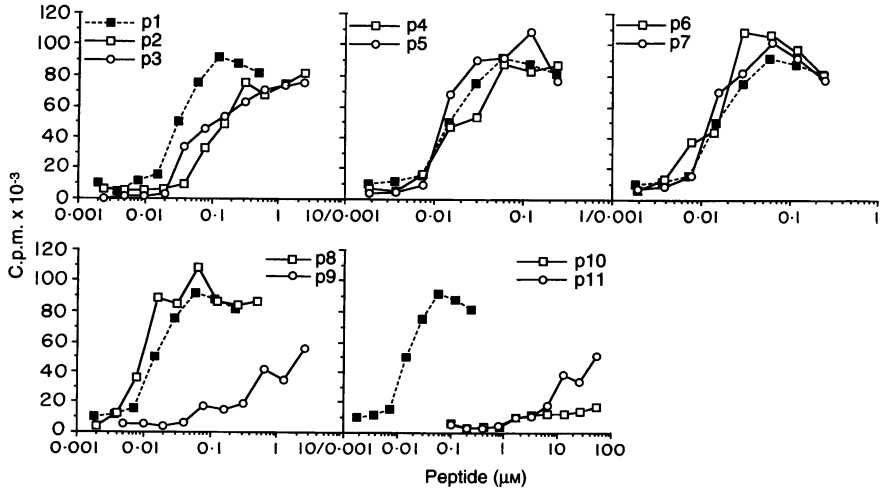

Figure 2. T-cell stimulatory capacity of poly(Ala)-substituted peptides. A $CD4^+$ T-cell line derived from rGST-19-immune LNC was stimulated (2×10^4 cells/well) with poly(Ala)-substituted peptides (0·001–50 μM) in the presence of irradiated syngeneic (C57BL/10) APC (3×10^5 cells/well). Data are from a single experiment that was repeated three times with similar results, and are presented as c.p.m. $\times 10^{-3}$ following 3 days of *in vitro* culture. Background counts (cells without antigen) were 2356.

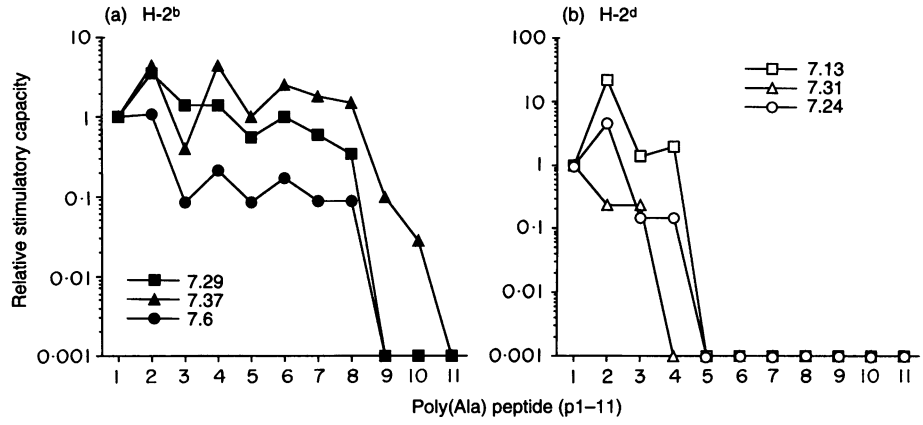

Figure 3. Recognition of poly(Ala)-substituted peptides by p61–80-specific T-cell hybridomas. Peptide 1-specific T-cell hybridomas, derived from C57BL/10 (H-2^b; clones 7.6, 7.29 and 7.37; closed symbols) or BALB/c (H-2^d; clones 7.13, 7.24 and 7.31; open symbols) mice were stimulated (2×10^4 cells/well) with poly(Ala)-substituted peptides (0·01–25 μM) in the presence of irradiated syngeneic APC (3×10^5 cells/well). Data are presented as relative stimulatory capacity [= ratio of concentration of the unsubstituted wild-type p1 (residues 61–80) required for 50% maximal response to the same value determined for the analogue peptide]. Background c.p.m. (cells

Immunogenicity of poly(Ala)-substituted peptides

In the following experiments, C57BL/10 (H-2^b) mice were primed with each of the peptide analogues (p1–11) and the draining LNC subsequently challenged *in vitro* with the homologous peptide or the wild-type p1. Several important points emerged from the analysis of immunogenicity and cross-reactivity of wild-type and analogue peptides, as shown in Fig. 5.

The immunogenicity of each analogue peptide, as determined by *in vitro* proliferative responses of primed T cells to an optimal concentration (25 μM) of homologous peptide, was retained, irrespective of the degree to which flanking residues were substituted (Fig. 5a).

Immunization with the wild-type p1 and challenge *in vitro* with each analogue peptide (Fig. 5b) resulted in a cross-reactive stimulation of potency comparable to the homologous response for p2–6, followed by a gradual decline down to p11, which was negative. T cells from mice immunized with PBS in IFA failed to respond, indicating that none of these peptides was mitogenic under the assay conditions used.

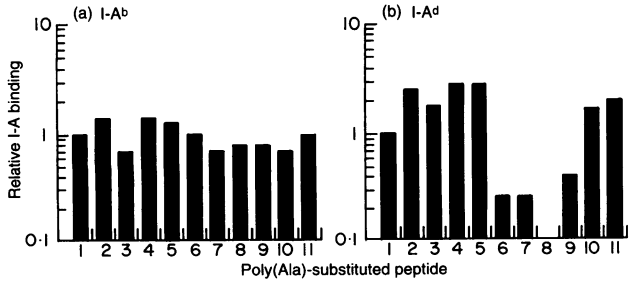

Figure 4. Binding of poly(Ala)-substituted peptides to H-2 I-A molecules. Poly(Ala)-substituted p1–11 were tested for their ability to inhibit the binding of biotinylated reference peptides (100 μM), as described in the Materials and Methods. Results are presented as relative binding to I-A^b and I-A^d compared with the binding of the unsubstituted p1. The IC50 of p1, before normalization, was 230 μM for I-A^b and 326 μM for I-A^d. The values shown represent the means of triplicate wells from a single experiment, which was repeated three times. Variations were < 20% of the mean.

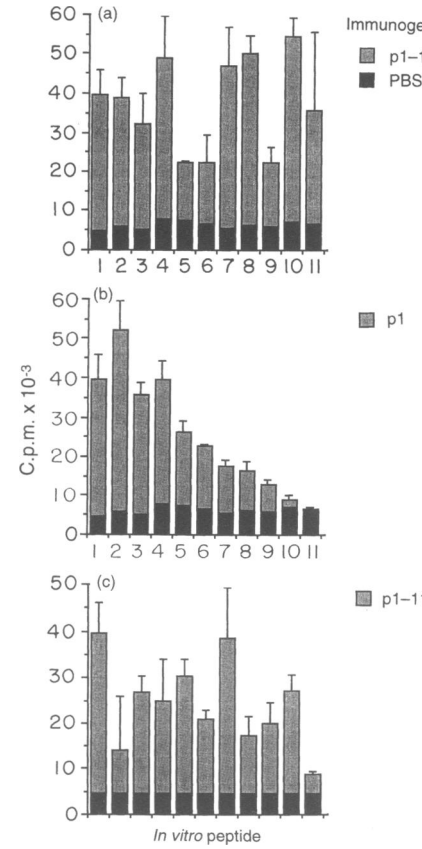

Figure 5. Immunogenicity of poly(Ala)-substituted peptides. Lymph node cells from C57BL/10 mice, immunized with 30 nmol p1–11 or PBS, as indicated in the figure legend, were cultured (4×10^5 cells/well) *in vitro* with 25 μM homologous peptide (a), p1–11 (b) or p1 (c) for 3 days, and thymidine incorporation determined during the last 6 hr of culture. Results are presented as c.p.m. $\times 10^{-3}$ and background c.p.m. of all peptide-immune LNC cultured in absence of antigen were < 5000.

The reciprocal experiment, i.e. immunization with each of the analogues and challenge *in vitro* with the wild-type p1 (Fig. 5c), resulted in a much broader cross-reactivity, as variable but significant proliferative responses were obtained after immunization with any analogue from p2 to p10. Interestingly, there was a clear correlation between the reciprocal cross-reactivities for p3–6, while for the remaining peptides cross-reactivities were vectorial if they existed.

Neither immunogenicity nor cross-reactivity showed a clear inverse relationship with respect to the number of alanine substitutions in the peptide although, as mentioned above, the results presented in Fig. 5b did show a general correlation in this respect. Thus, p7-immune cells, for example, cross-reacted more strongly with the wild-type p1 than did p6, despite it being more substituted (Fig. 5c).

DISCUSSION

In this study we have used a series of poly(Ala)-substituted peptide analogues to investigate the role of residues flanking the permissively recognized p69–76 core T-cell epitope from the 19 000 MW protein of *M. tuberculosis*. The capacity of analogue peptides to stimulate *in vitro* proliferative responses was tested initially with a 19 000 MW protein-specific T-cell line, revealing that all but one N-terminal flanking residue (Thr68) could be collectively replaced by alanine without significantly affecting peptide antigenicity. However, p9, with all flanking residues replaced with alanine, was ~100 times less potent than the control unsubstituted (p1) peptide. The only difference between the strongly antigenic p8 and p9, in terms of sequence, was substitution of Thr68 → Ala. However, as Thr68 alone is not required critically for recognition, as determined by single alanine substitution,[5] it is likely that the lack of potency observed with analogue p9 was due to the cumulative effect of substitutions within flanking residues. Furthermore, as analogue p9 retained the capacity to bind I-A^b molecules (Fig. 4a), this failure to stimulate must be TCR-mediated.

These results indicate that the effects observed with single, as opposed to multiple, alanine substitutions can be quite different and, furthermore, that the assignment of particular residues as 'critical' is, to a considerable extent, dependent upon the nature of the surrounding residues. Examples of functional interactions between amino acids that directly affect peptide recognition have been reported.[18] It was shown that the 'critical' role of an amino acid residue can be due to its interaction with a neighbouring 'non-critical' residue and it was suggested that the role of 'critical' residues may extend beyond contacting the TCR or Ia molecule, to include other functions such as stabilizing peptide conformation.[18]

The most striking finding to emerge from this study is the demonstration of significant differences between peptide-specific T-cell hybridomas of H-2^b and H-2^d haplotypes in terms of their requirements for flanking residues (Fig. 3). T hybridomas derived from C57BL/10 (H-2^b) mice responded effectively to analogues p1–9/p10, indicating that provided peptide length is maintained all but one of the N-terminal native flanking residues, as well as the three C-terminal flanking residues, can be replaced by alanines, without disrupting recognition of the epitope core. In contrast, the recognition of peptide analogues by T hybridomas derived from BALB/c (H-2^d) mice was clearly more dependent on the presence of the native flanking residues. The observed differences between H-2^b and H-2^d hybridomas may in part be explained by alleleic differences in peptide–MHC binding. Thus, the lack of stimulation observed with analogues p6–9, with BALB/c hybridomas, was associated with a partial decrease in I-A^d binding. However, the differential response of hybridomas to analogue p5 cannot be explained by this mechanism as p5 clearly bound both I-A molecules effectively. After the significant decrease in binding of p6–8 to the I-A^d molecule, additional substitutions (p9, p10, p11) led to a paradoxical recovery of the binding affinity. This finding may be explained by the fact that additional alanine substitutions may have inadvertently introduced a new I-A^d-binding motif.[19] Thus p11 [$(A)_9$LAANANIA$(A)_3$] bound to purified I-A^d molecules with similar affinity as the wild-type peptide (IC_{50} values as calculated for the data presented in Fig. 4 were 29·7 μM and 46·6 μM, respectively), whereas a peptide substituted at the putative p2 'anchor' position [$(A)_9$LAAAANIA$(A)_3$] bound I-A^d with ~13 times lower affinity (IC_{50}, 610 μM). The greater requirement for flanking residues by BALB/c-derived hybridomas is consistent with previous results showing significant differences between H-2^b and H-2^d haplotypes in terms of the minimal peptide length required to stimulate T-cell proliferation.[5] Irrespective of the mechanism that accounts for these differences, we believe the effects are most probably H-2 associated.

Although the poly-substituted analogue peptides used in our study were not designed for the purpose of identifying MHC class II 'anchor' residues, neither the wild-type p61–80 nor any analogues contained a recently identified I-A^b-binding motif.[20] Nevertheless, all analogues bound to the I-A^b molecule with relatively high affinity, implying either that this motif is not an absolute requirement for binding, or that positive interactions along the length of the peptide backbone make a significant contribution to the overall binding affinity, as suggested previously.[21,22] Furthermore, as most naturally occurring peptides are likely to contain amino acids that exert negative effects on binding,[22] poly(Ala) substitution may be a useful means of increasing peptide binding affinity without interfering with residues involved in T-cell specificity.

We also attempted to correlate the information obtained from *in vitro* experiments (MHC binding and proliferation) with the capacity of analogue peptides to generate immune responses *in vivo*. It is clear from the immunogenicity and cross-reactivity data shown in Fig. 5 that alanylated flanking regions are capable of stabilizing the immunogenic peptide and inducing strong T-cell responses, highly cross-reactive with the native peptide. Moreover, the results suggest that these properties cannot be predicted simply on the basis of sequence similarities, as immunization with p2 resulted in less cross-reactivity than with many other analogue peptides.

Analysis of poly(Ala)-substituted peptides from the amino-terminal epitope of rat myelin basic protein showed that reactivity with specific T cells was preserved when substitution affected all but five key residues of the native undecapeptide,[23] whereas substitutions of some of these key residues within the epitope core resulted in peptides that interfered with induction of the disease.[23,24] Moreover, other immunogenic epitopes have been highly substituted in both flanking and core residues without losing their capacity to bind class II molecules, as is

the case for an influenza haemagglutinin peptide binding to HLA-DR1 molecules[21] and a polysubstituted tetanus toxoid peptide.[25] In the present study we extend this information to a genetically permissive T-cell epitope, revealing that tolerance of substitutions within flanking residues is influenced by H-2 haplotype.

The results reported here suggest that a permissive epitope, such as the one selected for this study, tolerates considerable structural changes without impairment of its immune potential. It would appear to be a good candidate for exploring synthetic strategies requiring substantial modifications of the primary structure, although optimal specific responses require preservation of the core region. Nevertheless, the importance of flanking regions for the overall characteristics of immunogenic peptides has been found to be greater for other specificities where substitutions have resulted in significant differences in T-cell immunogenicity.[7,8] The nature of these flanking regions may also contribute to the importance of peptide orientation on the immunogenicity of hybrid peptides,[26,27] bearing in mind that optimization of flanking regions for T-cell epitopes should consider not only MHC binding and specificity, but also enzymatic processing of the peptide, as discussed, for instance, in relation to the specificity of cathepsins.[28]

Finally, recent studies have shown that even relatively subtle structural changes within peptide ligands can modulate the quality of the response obtained, resulting, for instance, in a different cytokine profile[29] or induction of anergy.[30] Whether such effects can also be mediated by changes in flanking residues is not known, but suggest that measurement of responsiveness by analysis of other parameters of T-cell function should be considered in future studies.

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