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Phil. Trans. R. Soc. Lond. B 1989 **323**, 553-564
doi: 10.1098/rstb.1989.0035

Phil. Trans. R. Soc. Lond. B **323**, 553–564 (1989)
Printed in Great Britain

Recognition of the HLA class II–peptide complex by T-cell receptor: reversal of major histocompatibility complex restriction of a T-cell clone by a point mutation in the peptide determinant

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Recognition of the HLA DR–peptide complex by an influenza haemagglutinin-specific T-cell clone was examined by assaying a variety of peptide analogues for their ability to be recognized. Consistent with earlier experiments arguing that the peptide blinds the restriction element in a helical conformation, acetylation of the amino terminus and amidation of the carboxy terminus of the natural determinant (residues 307–319) resulted in a peptide that exhibited both greater propensity to form a helix, as judged by circular dichroism, and the ability to stimulate the clone at concentrations approximately two orders of magnitude lower than the native sequence. The peptide was modelled into the potential antigen-combining site of HLA class II based on the ability of analogues containing point mutations to stimulate the T-cell clone. The working model was initially tested by examining the ability of Epstein–Barr-transformed B-cell lines expressing in different DR4 subtypes to present the native haemagglutinin sequence and analogues to the clone. The different alleles could be categorized as high, intermediate, or low responders based on the resulting proliferation. DR4 *dw*15 was a high-responding allele, *dw*4, 13, and 14 were intermediate-responding alleles, whereas *dw*10 was a low responder. Mutation of Gln to Arg at 312 in the haemagglutinin sequence converted the high and intermediate responders to non-responders, while turning the low-responding allele into an intermediate responder. Potential explanations for these effects are discussed in the context of the model of the complex between peptide and the major histocompatibility complex.

Introduction

The recent demonstration that peptide fragments of immunogenic proteins are bound by major histocompatibility complex (MHC) class II molecules has provided an important explanation for the genetic basis of immune responsiveness (Babbitt *et al.* 1985; Guillet *et al.* 1987; Buus *et al.* 1986). Although not the sole factor, the formation of a peptide–MHC complex appears to be a necessary requirement for both the generation of a cellular immune response and the T-cell dependent humoral response (reviewed by Moller 1987). Even though these experiments have generated a unified model of T-cell recognition, not all issues have been resolved. One confusing question is if binding to one of an individual's MHC proteins is critical to the generation of an immune response and subsequent protection from infection, how can a very limited

number of binding sites interact with a sufficiently large percentage of peptides from the multitude of proteins from pathogens to protect the individual? The ability of an MHC protein to interact with manifold peptides is quite different from the specificity characteristic of other known membrane receptors. In contrast with receptors of the endocrine system, which have evolved to optimize their specific interaction with ligand, the MHC proteins appear to have developed to bind a diverse range of ligands.

A possible explanation is that peptides that bind the MHC proteins and are recognized by T-cell receptors are limited in their diversity. Such a proposal has been supported by the structural similarities found in the defined helper and cytotoxic T-cell determinants (Rothbard & Taylor 1988). Potential allele-specific motifs can also be seen as the number of determinants has increased. At least two general patterns can now be identified. The most common are homologous residues found in relative positions that, if the peptide adopts a helical conformation, would constitute a face of the helix (examples are shown in figure 1 *a*, *b*). The similarities can be optimized if selected sequences are reversed, implying that some determinants could bind in the opposite direction to others. The second pattern is less common, and appears to be less compatible with a helical peptide. It is composed of five amino acids, the first being a charged amino acid, followed by two hydrophobic amino acids, then a proline, concluding with a polar amino acid (figure 1 *d*).

To determine whether these structural similarities have any merit, several different strategies have been used to examine the significance of a putative motif for DR1-restricted determinants. The initial experiments examined the shared pattern between the defined I-E^k, E^d and DR1 determinants (Rothbard & Taylor 1988). Successful presentation of peptide antigens to DR1-restricted T-cell clones by L cells expressing either the murine I-E α-chain and the DR1 β-chain or the intact murine I-E^k molecule demonstrated significant structural similarity in the antigen-combining sites of these two class II molecules (Lechler *et al.* 1988).

The simple motif of a positively charged amino acid and three hydrophobic residues (figure 1) has also been used to define previously unidentified DR1-restricted determinants in the ragweed E protein and the 19 kDa protein of *Mycobacterium tuberculosis* (Rothbard *et al.* 1988; Lamb *et al.* 1988). In the latter case, recognition of the peptide in association with DR1 and 4 was confirmed by using T-cell clones and transfected murine L-cell lines expressing DR molecules. As impressive as these predictions were, not all peptides containing this sequence motif will be guaranteed to be restricted by DR1 and 4, most likely because these four amino acids do not represent all the contact points between the peptide and the MHC protein. Nevertheless, the experiments provided further support for the importance of these residues in DR1- and DR4-restricted recognition and represent the potential of using detailed structural requirements to identify determinants *a priori*.

The third approach for studying the molecular requirements for recognition by DR1 was based on the assumption that if the structural similarity found in DR1-restricted determinants is involved in binding to the MHC protein, then the two influenza peptides, shown in figure 1 *b*, might interact with similar DR1 residues, and also adopt an identical conformation and be localized in the same position in the binding site. Consequently, residues interacting with DR1 should be able to be exchanged between the determinants without loss of recognition. The experiment presumes that such an exchange will not prevent the hybrid peptides from adopting the correct conformation by removing important intrapeptide interactions.

For each T-cell clone, a hybrid peptide, created from such exchanges based on the alignment

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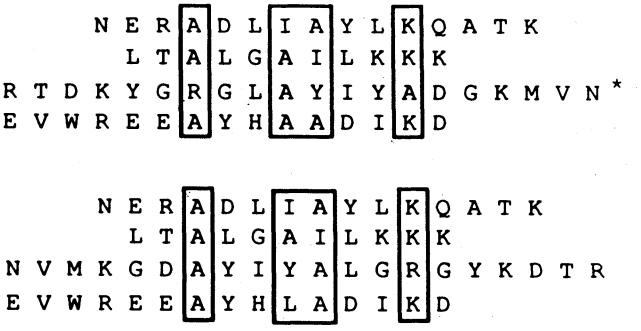
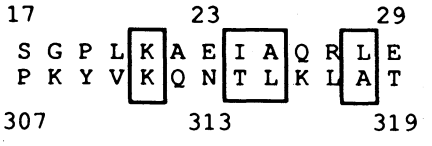
(*a*) E^k-restricted determinants

cytochrome moth 89–103
sperm whale myoglobin 69–78
staphylococcus nuclease 81–100
ragweed allergen 51–65

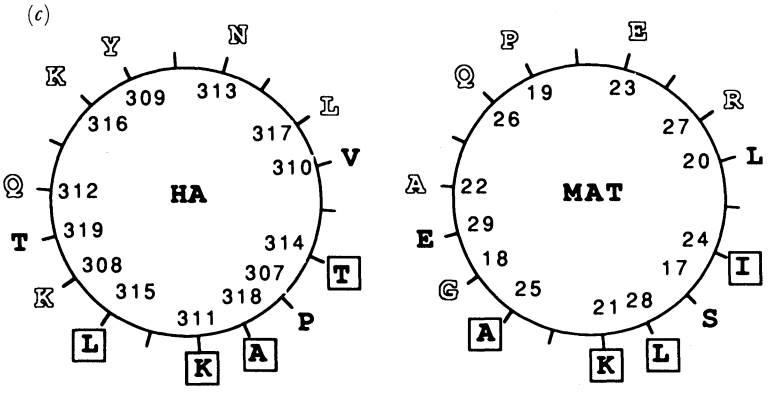


(*b*) DR1-restricted determinants

influenza matrix protein 17–29
influenza haemagglutinin 307–319



(*c*)



(*d*)

influenza haemagglutinin 257–266
murine cytomegalo. virus pp 89 167–176
human acetylcholine receptor 257–269

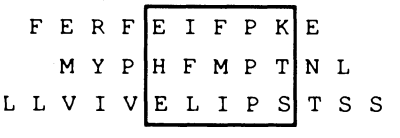


FIGURE 1. Structural similarities found in selected T-cell determinants. (*a*) The sequences of four E^k-restricted T-cell determinants (Heber-Katz *et al.* 1983; Livingstone & Fathman 1987; Finnegan *et al.* 1986; Kuisaki *et al.* 1986) aligned to emphasize structural similarities at relative positions 1, 4, 5, and 8 (enclosed in boxes). If selected sequences (★) are reversed, similarities can be enhanced. (*b*) Two DR1-restricted determinants aligned to reveal structural similarity. (*c*) Two DR1-restricted determinants displayed on helical wheels demonstrating that the residues enclosed in boxes in (*a*, *b*) constitute the face of a helix. The outlined amino acids could be exchanged between determinants with maintenance of T-cell recognition (Rothbard *et al.* 1988). (*d*) Selected T-cell determinants oriented to demonstrate sequence similarity around a proline. The haemagglutinin determinant is E^d restricted (Hackett *et al.* 1983), the murine cytomegalo-virus determinant is L^d restricted (Del Val *et al.* 1988), whereas the acetylcholine receptor peptide is DR3 restricted (Brocke *et al.* 1988).

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shown in figure 1, was stimulatory (Rothbard *et al.* 1988). Substituting six residues from the haemagglutinin peptide (printed as hollow characters in figure 1*c*) for amino acids in the corresponding positions in the matrix sequence resulted in a peptide that was recognized by the haemagglutinin-specific clone. However, an equivalent proliferative response required approximately one hundred times as much peptide as the natural sequence. The reciprocal exchange of six residues did not result in a stimulatory peptide for the matrix-specific clone. A possible explanation was the presence of two prolines in the hybrid peptide, which was hypothesized to interfere with its ability to adopt a helical conformation. Replacement of one (corresponding to 307 in the haemagglutinin sequence), but not the other (Pro 19 in the matrix peptide), with Ala resulted in a peptide that could stimulate the matrix-specific clone.

As can be seen in the simple helical wheel representation, the substitutions necessary to generate stimulatory peptides constituted the complete upper facade of the peptide. Our interpretation was that only if an intact constellation of residues interacting with both the MHC protein and the antigen receptor of the T-cell were present would the clone be stimulated. Point mutations at critical positions in either set of amino acids can prevent the peptide from being recognized.

We examine below both the proposed conformation and location of the haemagglutinin (HA) peptide in the DR1-binding site.

Methods

Isolation of antigen-reactive T-cell clones

The isolation and characterization of human T-lymphocyte clones HA 1.7 have been described previously (Lamb *et al.* 1982*a*, *b*).

Proliferation assays

Cloned T cells (5×10^4 ml^{-1}) were cultured with soluble antigen in the presence of irradiated histocompatible peripheral blood mononuclear cells (1.25×10^5 ml^{-1}), autologous Epstein–Barr virus- (EBV-)transformed B cells (10^5 ml^{-1}), or mitomycin-C-treated transfected murine L cells (10^5 ml^{-1}) in a total volume of 200 µl of complete medium in 96-well round-bottom plates. After 72 h of incubation, tritiated methyl thymidine (1 µCi†, [^{3}H]TdR; Amersham International, Amersham, U.K.) was added to the cultures for 8–16 h and then harvested onto glass-fibre filters. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute (c.p.m.) plus or minus percentage error of the mean for triplicate cultures.

Peptide synthesis, analysis, and purification

Peptides were synthesized by using solid-phase techniques (Barany & Merrifield 1979) on an Applied Biosystems 430A peptide synthesizer utilizing commercially available reagents as described previously (Rothbard *et al.* 1988). The purity of the peptides were assayed by using amino acid analysis and high pressure liquid chromatography on an Aquapore RP-300 reverse-phase column (Brownlee Laboratories). Each peptide was purified on a RP-300 column with a water–acetonitrile–trifluoroacetic acid gradient before circular dichroism

† 1 Ci = 3.7×10^{10} Bq.

measurements. The structures of the analogues were confirmed by using fast atom bombardment mass spectroscopy.

Circular dichroism measurements

Circular dichroism measurements were made at room temperature of peptides (0.15 mg ml^{-1}) dissolved in differing mixtures of water and trifluoroethanol on a Jasco 41C spectropolarimeter equipped with a model J-D DPY data processor. The values shown are a result of an average of at least three scans.

Results and discussion

Modification of the peptide to improve potency

If the haemagglutinin peptide binds the restriction element in a helical conformation, then stabilization of this conformation might improve the potency of the peptide. Recent experiments have demonstrated that stabilization of the macrodipole of an α-helix can result in a significant increase in the helical content of relatively short peptides (Shoemaker *et al.* 1987). One way to stabilize the macrodipole is to remove the charges at the amino and carboxy termini of the peptide by acetylation and amidation.

The natural haemagglutinin peptide and several analogues were examined for secondary structure content by circular dichroism in a range of water–trifluoroethanol (TFE) mixtures. The unacetylated and unamidated haemagglutinin peptide exhibited only low helicity even at high TFE concentration (figure 2*a*) as judged by absorption of circularly polarized light at 222 nm. Amidation and, to a greater extent, combined acetylation and amidation substantially increased the helicity of the haemagglutinin peptide. The peptides underwent simple helix–coil transitions, with helicity increasing with greater TFE concentration. The transition was not concentration dependent (data not shown) suggesting that aggregation is not important in the induction of the α-helix.

Analogues containing alternative amino acids for Pro 307 also were examined for greater helical propensity. Replacement of the Pro at 307 with Arg does not affect the ability of the peptide to form a helix. However, amidation and acetylation does. Consistent with the hypothesis that the improved helical content is caused by stabilization of the macrodipole, acetylation and amidation of the Arg-containing peptide results in greater helical content, but not as much as when the positively charged Arg is not at the amino terminus.

When tested in proliferation assays with the T-cell clone HA 1.7, the acetylated and amidated peptide can stimulate the clone at concentrations approximately two orders of magnitude lower than the natural sequence (figure 2*b*). Replacement of the amino-terminal Pro by either Lys or Arg also results in more potent peptides (figure 2*b*), but the acetylated, amidated peptide containing Arg at 307 is only recognized as well as the natural sequence acetylated and amidated (data not shown). That the effects of exchanging Pro for a positive charged residue and blocking the charged end groups are not additive indicates that the two factors are acting by separate mechanisms. The most likely explanation is that a positive charge at 307 results in a superior interaction with a residue in the binding site of DR1, but as shown, does not stabilize the macrodipole.

Even though these results can be interpreted as being due to the increased propensity of the peptide to be helical, an alternative explanation is that by removing the end-charge groups a

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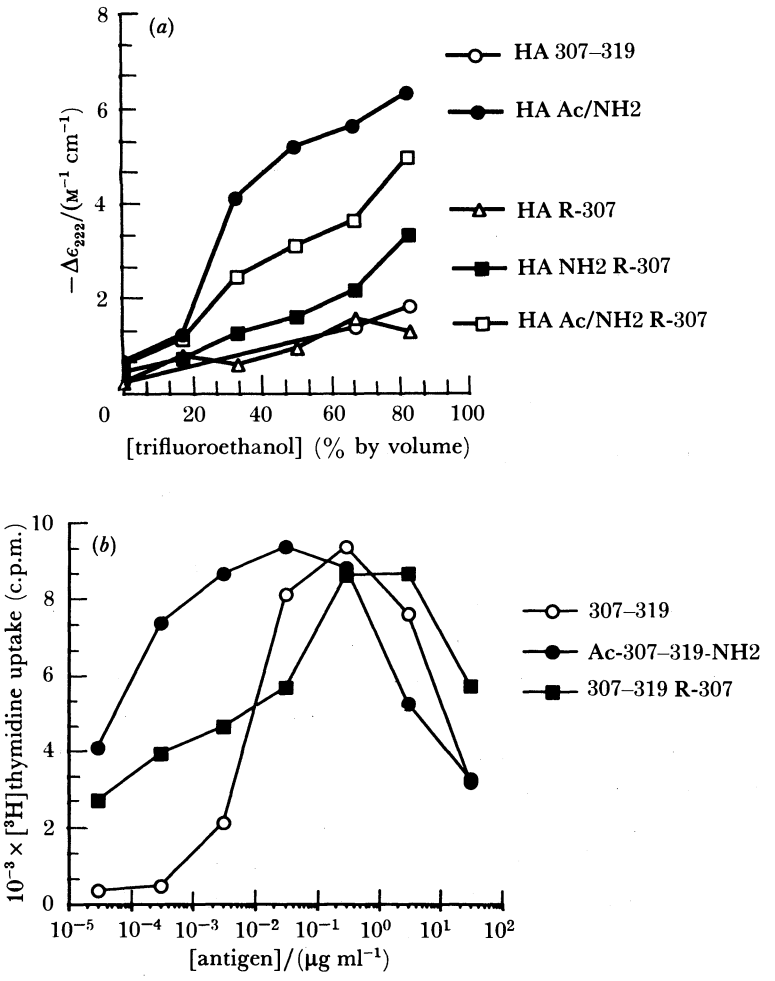

FIGURE 2. Increased potency of analogues of HA 307–319 correlates with greater propensity of the peptide to form a helix. (*a*) Differences in absorption between left and right circularly polarized light at 222 nm for HA 307–319, HA 307–319 acetylated and amidated (HA Ac/NH_2), HA 307–319 with Arg at 307 (HA R–307), HA 307–319 with Arg at 307 only amidated (HA NH_2 R–307), and HA 307–319 with Arg at 307 both acetylated and amidated (HA Ac/NH_2 R–307) in differing water–trifluoroethanol mixtures. On this scale, a circular dichroism value of 10 is conventionally taken to be equivalent to 100% helix. (*b*) Proliferation of HA 1.7 in response to HA 307–319, HA 307–319 acetylated and amidated (HA Ac/NH_2), and HA 307–319 with Arg at 307 (HA 307–319 R–307).

peptide is created that can bind the restriction element more effectively than the natural sequence. Because these two issues are so closely related, they cannot yet be easily distinguished.

Identification of specific contact residues in the DR1-binding site

If the proposed model of the orientation of peptide relative to the two macromolecules is valid, complementary residues in the binding site to those of the peptide should be able to be identified. To identify aspects of the peptide sequence that were critical for interaction with DR1 and the T-cell receptor, peptide analogues of the haemagglutinin peptide containing point mutations were synthesized and assayed for their ability to stimulate the T-cell clone.

To identify an approximate position of the peptide in the antigen-combining site of DR1, a model of DR1 was generated based on the published HLA A2 structure (Bjorkman *et al.*

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1987*a*, *b*) (figure 3*a*). As previously detailed (Brown *et al.* 1988) alignment of the MHC class I and II alleles, whose sequence has been determined, reveal that the two classes of proteins share a number of structural features. In particular, the antigen-combining sites of both are composed of identical secondary structural units, even though the class II site is composed of residues from both the α- and β-chains whereas class I is generated by the folding of a single

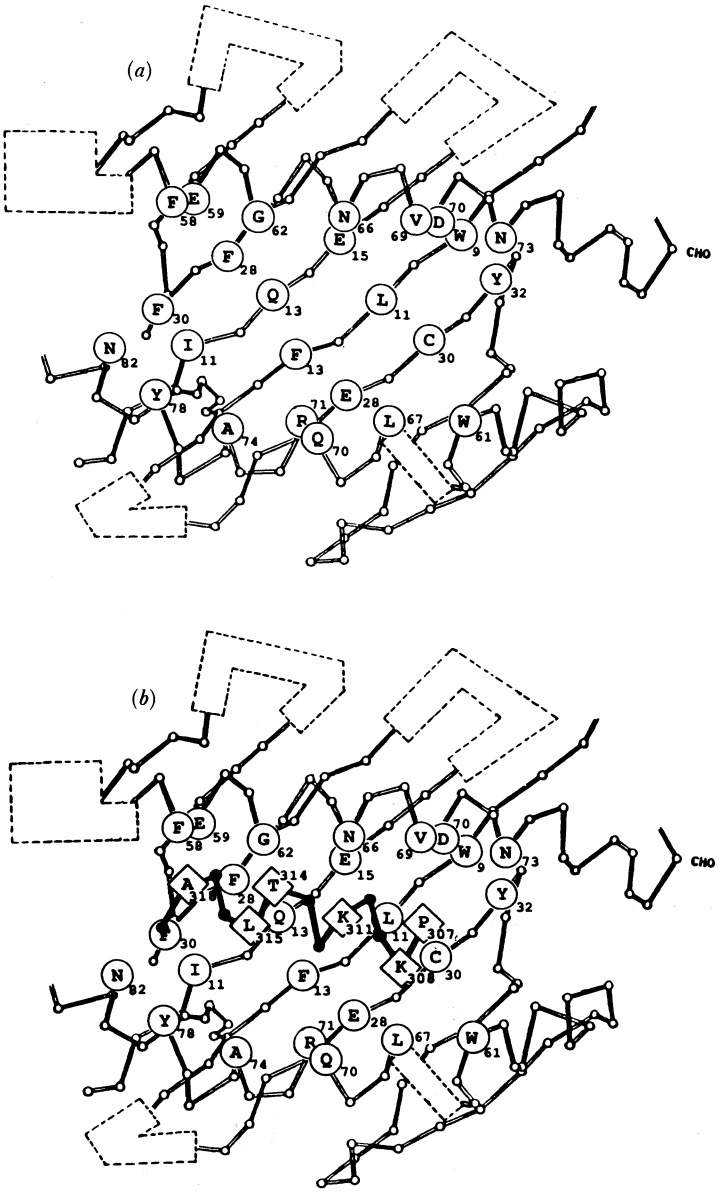

FIGURE 3. Schematic representation of the amino-terminal domains of the α- and β-chains of HLA DR1. (*a*) The proposed antigen-combining site of DR1 represented as initially done by Brown *et al.* (1988). The model is based on the crystal structure of HLA A2. The areas enclosed in dashed lines correspond to positions with either additions or deletions of residues between consensus class I or II sequences. Residues enclosed in circles are believed to point into the putative antigen-combining site. (*b*) A potential location of HA 307–319 in the combining site. The peptide is modelled as three turns of a helix and placed in the site antiparallel with the α-chain helix. The orientation of the peptide relative to the MHC protein and the antigen receptor is consistent with the experiments exchanging residues between determinants (Rothbard *et al.* 1988). The residues of the peptide believed to be facing down in the site are enclosed in diamonds.

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polypeptide chain. In addition, many of the residues exhibiting sequence variation between alleles can be placed in similar positions in the binding site for both class I and class II MHC proteins.

Generating a working model of the peptide ligand in the proposed binding site involved several major assumptions. We assumed that the antigen receptor of the T-cell clone recognizes the peptide in an unique conformation and location in the site, making specific contacts with the residues composing the cleft. These assumptions do not preclude the possibility that the peptide might be able to bind to DR1 in several different locations and perhaps even with different conformations, but these are assumed not to be recognized by the antigen receptor of this particular clone. The previous helix-exchange experiments were interpreted to have established that the peptide bound in a helical conformation. In the orientation shown in figure 1*c*, the residues forming the lower face of the helix are believed to interact with DR1, whereas the residues on the upper facade contact the T-cell receptor.

Based on analyses of helices packing onto β-pleated sheets (Cohen *et al.* 1982; Janin & Chothia 1980; Chou *et al.* 1985) a 13-amino-acid peptide, folded as three turns of an α-helix, can bind in a limited number of ways. Our goal was to generate a working model of the peptide interacting with specific residues in the site, both to understand MHC restriction and to increase binding affinity. Even though we shall speak of potential contacts and even postulate a preferred location of the peptide in the binding site, the details of binding can only be approximated, both because of the uncertainty of the class II model and the flexibility of the sidechains of both the restriction element and the ligand.

The dimensions of the proposed antigen-combining site are sufficient to bind a helical peptide with its helical axis approximately parallel with those of the α_1-helix of class I or the helix of the α-chain of class II, which form one side of the cleft. In such a model, the residues composing the lower facade of the helical peptide would be expected to closely pack onto the four central strands of the β-pleated sheet of the restriction element. A potential diversity of molecular contacts can result because of the length and flexibility of the amino acid sidechains of both the MHC molecule and the peptide. Thus the corresponding amino acids of two different peptides bound in the same location in the site might not necessarily interact with the identical residues of the β-strands. The amino acids of the determinant forming the sides of the helix could interact with corresponding residues of the helices of the α- and β-chains of DR1. Steric constraints also will be important, and size limitations were apparent in modelling the helix–helix contacts.

The degeneracy of restriction of the haemagglutinin-specific T-cell clone for both DR1 and 4 (Eckels *et al.* 1984) allowed us to use the natural variants of DR4 to help localize the peptide in the site. EBV-transformed B cells representative of the DR4 *dw* subtypes were used to present HA 307–319 to the cloned T cells. Marked variations in the efficiency of antigen presentation were observed with the subtypes were compared. B cells expressing *dw*4 and *dw*15 were indistinguishable from cells expressing the parental restriction element DR1 (table 1). However, *dw*13, 14, and more dramatically *dw*10 were less effective in presenting antigen. Cells expressing *dw*13 and *dw*14 were capable of eliciting approximately 25% of a response generated by the high-responding alleles, whereas *dw*10 elicited less than 10% of the maximal response at 3 μg ml^{-1}. The differences cannot be accounted for by variation in the level of cell-surface DR, because by flow cytometric analysis the DR density on all the EBV–B cells was not the result of differential processing.

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Table 1. Partial primary sequence of the β-chains of DR1 and DR4 *dw*15

(Gregerson *et al.* (1986) and Bell *et al.* (1987).)

									10									20								30							
DR1	G	D	T	R	P	R	F	L	W	Q	L	E	F	E	C	H	F	F	N	G	T	E	R	V	R	L	L	E	R	C	
DR4 *dw*15	–	–	–	–	–	–	–	–	–	E	–	V	–	H	–	–	–	–	–	–	–	–	–	–	–	F	–	D	–	Y		
									40									50								60						
DR1	I	Y	N	N	Q	E	E	S	V	R	F	D	S	D	V	G	E	Y	R	A	A	T	E	L	G	K	P	D	A	E		
DR4 *dw*15	F	H	Y	–	–	–	–	Y	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–				
									70									80								90						
DR1	Y	W	N	S	Q	K	D	L	I	E	Q	R	R	A	A	V	D	T	Y	C	R	H	N	Y	G	N	G	E	S	F		
DR4 *dw*15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–		

If amino acid sequences of the β_1-domain of the DR4 subtypes are compared with DR1, there are multiple differences in the amino terminal half of the domain that contribute to T-cell antigen recognition (table 1). However, the sequence identity between the DR4 subtypes in this region proves that these differences cannot account for the functional variation seen between the *dw*4/15- and *dw*10-expressing cells. Only sequence differences present in the β_1-helix can account for the different abilities to present antigen. When the sequence differences between the DR4 *dw* subtypes are analysed, prominent variations between the low-responder allele, *dw*10, and the remaining subtypes occur at positions 70 and 71 (table 2). DR4 *dw*10 contains Asp at position 70 and Glu at position 71. In contrast, the other alleles have Gln at 70 and a positively charged residue at 71. In the working model of class II proteins, both positions are centrally located in the bent helix composing the side of the proposed antigen-combining site. Residue 70 has been modelled to point up towards the T-cell receptor whereas residue 71 points in towards the combining site.

Table 2. Differential response of cloned T cells to HA 307–319 presented by EBV-transformed B cells expressing the DR*dw* subtypes

(Culture conditions and proliferation were determined as described in Methods. Results expressed as change in counts per minute with the background response of T cells cultured with EBV-transformed B cells (less than 3500 c.p.m.) subtracted from proliferation observed when antigen is present. From Gregerson *et al.* (1986).)

HLA-DR specificity	amino acids at β-chain positions 57	67	69	70	71	74	86	proliferative response (Δ c.p.m.) (3 μg ml^{-1})
DR1	D	L	E	Q	R	A	G	34288
DR4 *dw*4	D	L	E	Q	**K**	A	G	22793
*dw*10	D	**I**	E	**D**	**E**	A	**V**	2435
*dw*13	D	L	E	Q	R	**E**	**V**	17554
*dw*14	D	L	E	Q	R	A	**V**	14562
*dw*15	**S**	L	E	Q	R	A	G	38416

The peptide can be placed in the binding site in either of two directions, either parallel or antiparallel with the α-chain helix. In the antiparallel direction, a location can be found with Lys 308 and Lys 311 next to two negatively charged amino acids in the binding site, Glu 15 of the α-chain and Glu 28 of the β-chain (figure 3*b*). In this orientation, residue 71 of the β-chain is directed at Gln 312 of the peptide. If the peptide binds DR4 in the same location as

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proposed for DR1, and if the low-responder phenotype arose from the inability of the peptide to interact with the restriction element, responsiveness might be recovered by mutating the peptide at position 312.

When Gln 312 was converted to a negatively charged amino acid, the peptide analogues were recognized by the T cells as well as the native peptide when presented by DR4 *dw*15 (figure 4*a*). However, these analogues failed to stimulate the cloned T cells when *dw*10 cells were used as presenting cells (figure 4*b*). In contrast, a peptide containing Arg at 312 stimulated the clone when presented by *dw*10, but not DR4 *dw*15 antigen-presenting cells. Interestingly, peptides containing Lys at 312 were able to be presented by both *dw*10 and *dw*15 EBV–B cells.

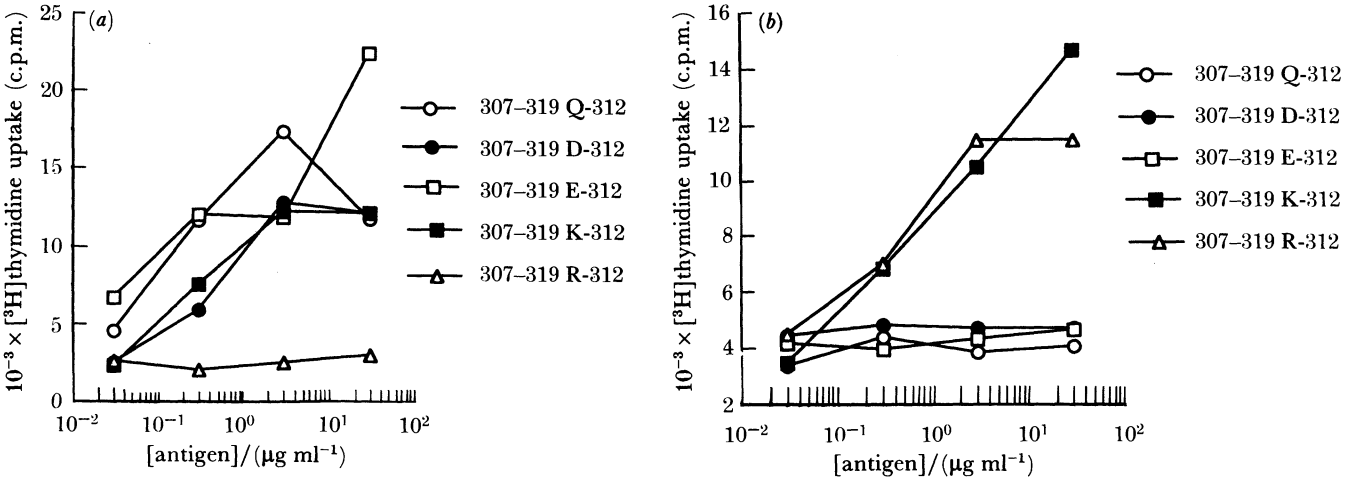

FIGURE 4. Recognition of analogues of HA 307–319 with mutations at residue 312 by HA 1.7 when presented either by (*a*) DR4 *dw*15 or (*b*) DR4 *dw*10 EBV-transformed B cells. Proliferation was measured as described in Methods.

The reversal of restriction of the T cell implies that residue 312 of the peptide interacts with residue 71 of the β-chain of DR4. However, before the interaction is proved, mutations at other positions in the peptide need to be analysed and additional binding and/or competition experiments need to be done. The results also demonstrate the subtle nature of the peptide–MHC interactions. There are significant differences between recognition of a peptide containing Arg and Lys at the same position. The differences can be rationalized by differences in the length, chemical character, and flexibility of the amino acid sidechains but the exact reason or reasons for the variations remains to be determined.

The functional importance of residues 70 and 71 in the β_1-helix in T-cell recognition has been demonstrated previously in murine T-cell responses restricted by I-A^b and the I-A^{bm12} mutants (Kanamori *et al.* 1984; Ronchese *et al.* 1987). Also, position 71 has been identified to be critical in the DR4-restricted T-cell responses found in rheumatoid arthritis patients (Todd *et al.* 1988).

The strategy of using mutant HLA molecules to present antigen and to complement the mutations with changes in the peptide appears to be an extremely useful method both for analysing the location of the peptide in the binding site and for examining the structural requirements for binding and recognition by T-cell receptors.

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We thank Steve Martin and Peter Bailey at the National Medical Research Laboratories at Mill Hill for help with the circular dichroism measurements, Kevin Howland and Vineeta Bal for excellent experimental assistance, and Wendy Senior in helping prepare the manuscript.

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Discussion

B. H. Nicholson (*Department of Physiology and Biochemistry, University of Reading, U.K.*). Has Dr Rothbard observed any preference for *particular* hydrophobic residues in his predictive motif, as opposed to hydrophobic residues in general?

J. B. Rothbard. The observed frequencies are documented in Rothbard & Taylor (1988).

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