ANALYSIS OF PEPTIDE BINDING PATTERNS IN DIFFERENT MAJOR HISTOCOMPATIBILITY COMPLEX/T CELL RECEPTOR COMPLEXES USING PIGEON CYTOCHROME *c*-SPECIFIC T CELL HYBRIDOMAS

Evidence that a Single Peptide Binds Major Histocompatibility Complex in Different Conformations

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An individual has a limited set of MHC alleles, but has the capacity to respond to a broad range of different antigens. Thus, each MHC molecule has the capacity to bind a large number of diverse antigens (1), perhaps through a single binding site (2). In only a few cases (3–5), however, does a single antigenic peptide express degenerate restriction on a variety of unrelated class II MHC molecules.

Pigeon or moth cytochrome *c* (pcyt *c* or mcyt *c*)[1] show MHC restriction to various class II I-E molecules that have a conserved α chain (E^k_α) and the β chain of either the k, b, or s haplotype. There are only a few residue differences between these haplotypes, in the antigen binding site, in that the I-E^b_β and I-E^s_β chain differ from the I-E^k_β chain at four and seven amino acid sequence positions, respectively (6). The I-E^k-restricted T cell response to pcyt *c* is specific for a peptide within the COOH-terminal sequence 88–104 (7). A number of pcyt *c*-specific hybridomas have been shown to recognize the p88–104 and closely related m88–103 peptide, but have different specificities for the Eβ chain of the I-E molecule (8, 9), in that each hybridoma will recognize either the pigeon or moth peptide on at least two of three different I-E haplotypes. Thus, T cell hybridoma 22.D11 recognizes pcyt *c* on B10.A APC ($E^k_\beta E^k_\alpha$) and on B10.A(5R) APC ($E^b_\beta E^k_\alpha$), and has no response to mcyt *c* (8). T cell hybridoma 2H10 will respond to pcyt *c* on B10.A and B10.S(9R) APC ($E^s_\beta E^k_\alpha$), and 2B4 will respond to mcyt *c* on B10.A and B10.A(5R) APC, and pcyt *c* only on B10.A APC (10). We have taken advantage of the ability of these three different T cell hybridomas to recognize pcyt *c* or mcyt *c* peptides on these I-E molecules to examine which residues in these determinants are used to bind the I-E molecules.

Using functional assays, MHC binding sites (designated agretopes) and TCR

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[1] *Abbreviations used in this paper:* mcyt *c*, moth cytochrome *c*; pcyt *c*, pigeon cytochrome *c*.

binding sites (designated epitopes) have already been defined for some antigen/TCR systems, such as hen egg-white lysozyme (11) and for two similar pcyt *c*-specific T cell clones (12). We have extended these studies by analyzing the recognition of single residues in both pcyt *c* and mcyt *c* peptides on three different I-E haplotypes by three different T cell hybridomas. Using single amino acid-substituted peptide analogues of the above two antigens, we have addressed the following questions. Given that p88–104 and m88–103 are identical in their amino acid sequence, except for the deletion of an alanine residue at position 103, do these two antigens use the same residues as epitopes and/or agretopes? Is it possible that a single antigen can bind the MHC molecule in more than one conformation and, if so, what role does the TCR play in the binding pattern displayed by the peptide?

The interpretation of our results are based on criteria that define an epitope as a residue in the determinant that, when altered, renders the analogue nonstimulatory but still permits it to compete with the native peptide for MHC binding and an agretope as defined by Schwartz (7), i.e., a residue substitution that shifts the relative potency of the analogue when presented by different MHC molecules. In this paper we show that the reactivity patterns for the recognition of pcyt *c* and mcyt *c* by the three hybridomas, derived in this way, differ considerably, and that most residues within 95–104 or 95–103 are used as epitopes or agretopes by at least one of the TCR specificities. The distribution of these residues for each of the reactivity patterns does not fall into a helical configuration for any of the reactivity patterns observed, in contrast with studies on the T cell recognition of a lysozyme (11) and myoglobin (13) peptide. Indeed, our findings suggest that not only do the moth and pigeon peptides use a different set of agretopic residues to bind to the MHC molecules, but that each peptide may interact with each MHC molecule in different conformations. Thus, a single class II MHC molecule appears to be able to present the same peptide in different configurations, depending upon the TCR that makes up the ternary complex.

Materials and Methods

Mice. B10.A ($E^k_\beta E^k_\alpha$), B10.A(5R) ($E^b_\beta E^k_\alpha$), and B10.S(9R) ($E^s_\beta E^k_\alpha$) mice were bred in the vivarium at Scripps Clinic, La Jolla, CA.

Peptide Antigens. The synthetic peptides used in this study are shown in Figs. 1 and 2. They were either synthesized by the peptide synthesis facility of Scripps Clinic, under the direction of Dr. Richard Houghten, by a modified procedure (14) of the standard solid-phase peptide synthesis method (15), or in our laboratory as previously described (16). The crude products were extracted in 15% aqueous acetic acid, lyophilized, and subsequently purified to 98% purity by reverse-phase HPLC using a 1 × 25-cm Vydac C18 column (Sep/a/ra/tions Group, Hesperia, CA) and gradients of acetonitrile (0–50% in 30 min) containing 0.1% trifluoroacetic acid. Peptide compositions were verified using amino acid analysis.

Cell Lines. The B10.A anti-pcyt *c* T cell hybridomas, 2H10 (10) and 2B4 (17) were obtained from S. Hedrick, University of California, San Diego, CA. The B10.BR anti-pcyt *c* T cell hybridoma 22.D11 was produced in our laboratory (8). All three hybridoma lines were recloned for high specific activity before use. The subclones used in this study were 2H10.a, 2B4.49, and 22.D11/h. The IL-2 growth-dependent line CTLL, originally derived by Gillis and Smith (18), was obtained from S. Webb, Scripps Clinic. All the lines were maintained in complete medium containing DME (Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 10% NCTC-135 medium (Gibco Laboratories, Grand Island, NY), 5×10^{-5} M β-mercaptoethanol, 4 mM glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, and 6 mM Hepes.

Antigen-specific stimulation of T cell hybridomas was measured as the antigen-specific release of IL-2 into the culture supernatant using IL-2-dependent CTLL cells (19). Briefly, T cells (2×10^5) were incubated together with APC and various dilutions of antigen for 24 h at 37°C in 200 μl of complete media in 96-well flat-bottomed plates. Spleen cells (5×10^5/well) obtained from various strains were irradiated at 3,300 rad before they were used as APC. At the end of a 24-h stimulation period, 50 μl of each culture supernatant was removed and diluted to 200 μl with complete media containing 5×10^3 CTLL cells/well. These cells were cultured for 24 h at 37°C, pulsed with 1 μCi of [^{3}H]thymidine per well, and harvested 12 h later on glass-fiber filter discs using a Minimash cell harvester (M.A. Bioproducts, Walkersvile, MD). Averages (cpm) of triplicate determinants for each peptide concentration were plotted as dose-response curves from which the half-maximum values were calculated and compared with that of the native peptides pcyt *c* 88–104 and mcyt *c* 88–103.

Competition Assays. The analogues that were nonstimulatory at 100 μM/well in the direct stimulation assay described above were added together with the native peptide to the APC before the addition of T cell hybridomas. The competition assays were carried out with 100 μM/well of the analogue and the titration of the native peptide from 20 μM/well in the assay. The effect of the analogue on the T cell response to the native peptide was observed as either blocking or enhancement when a shift in the half-maximum value of the native peptide was observed. For some analogues there was no overall change.

Results

Three pcyt c-specific T Cell Hybridomas Show Marked Differences in their Fine Specificity for Sequence 95–104. The experiments were carried out with the three T cell hybridoma lines shown in Table I, which have previously been shown to have different specificities for MHC (I-E^k)-restricted pcyt *c* and mcyt *c* responses (8–10) T cell hybridoma 22.D11 recognizes pcyt *c* on B10.A APC ($E^k_\beta E^k_\alpha$) and on B10.A(5R) APC ($E^b_\beta E^k_\alpha$), and does not respond to mcyt *c* at all. T cell hybridomas 2H10 and 2B4 are also specific for pcyt *c*, but in addition, have a heteroclitic response to mcyt *c* on B10.A APC with degenerate MHC restrictions (10).

In previous reports from our laboratory (8, 20) and others (21), it was established that the minimal length of the pcyt *c* peptide required for T cell stimulation is the COOH-terminal sequence containing residues 95–104. However, the addition of residues to the NH_2 terminus of residue 95 increases the antigenic potency of the peptide; the maximal response is induced by pcyt *c* 88–104 (16). In the studies described in this paper, we have used single amino acid-substituted synthetic peptides of pcyt *c* (Fig. 1) and mcyt *c* (Fig. 2). All analogues had the same number of residues with the sequence conserved from residues 88 through 94, but with single site substitutions introduced from positions 95 to 103/104 for mcyt *c* and pcyt *c*, respectively.

TABLE I

The Definition of Functional Phenotypes for pcyt c-specific T Cell Hybridomas

		APC		
T cell hybrid	Functional phenotypes	B10.A ($E^k_\beta E^k_\alpha$)	B10.A(5R) ($E^b_\beta E^k_\alpha$)	B10.S(9R) ($E^s_\beta E^k_\alpha$)
2B4.49	I	P,M	M	-
2H10.a	IIIa	P,M	-	P
22.D11	IV	P	P	-

P, pigeon cyt *c*; M, moth cyt *c*.

	88																104
p88-104	K	A	E	R	A	D	L	I	A	Y	L	K	Q	A	T	A	K
K104R	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
A103K,K104A	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	A
K104A	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A
A103K	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	K
A103I	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	K
T102A	K	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	K
T102S	K	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	K
A101I	K	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	K
A101Y	K	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-	K
Q100N	K	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	K
Q100A	K	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	K
K99A	K	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	K
K99R	K	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	K
L98F	K	-	-	-	-	-	-	-	-	-	F	-	-	-	-	-	K
L98A	K	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	K
Y97A	K	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	K
Y97I	K	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	K
A96Y	K	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	-	K
A96I	K	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	K
I95A	K	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	K
I95Y	K	-	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	K

FIGURE 1. Peptide analogues of p88-104.

	88															103
m88-103	K	A	E	R	A	D	L	I	A	Y	L	K	Q	A	T	K
K103A	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A
T103R	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
T102V	K	-	-	-	-	-	-	-	-	-	-	-	-	-	V	K
T102A	K	-	-	-	-	-	-	-	-	-	-	-	-	-	A	K
A101I	K	-	-	-	-	-	-	-	-	-	-	-	-	I	-	K
A101Y	K	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	K
Q100N	K	-	-	-	-	-	-	-	-	-	-	-	N	-	-	K
Q100A	K	-	-	-	-	-	-	-	-	-	-	-	A	-	-	K
K99A	K	-	-	-	-	-	-	-	-	-	-	A	-	-	-	K
K99R	K	-	-	-	-	-	-	-	-	-	-	R	-	-	-	K
L98A	K	-	-	-	-	-	-	-	-	-	A	-	-	-	-	K
L98F	K	-	-	-	-	-	-	-	-	-	F	-	-	-	-	K
Y97A	K	-	-	-	-	-	-	-	-	A	-	-	-	-	-	K
Y97I	K	-	-	-	-	-	-	-	-	I	-	-	-	-	-	K
A96Y	K	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	K
A96I	K	-	-	-	-	-	-	-	I	-	-	-	-	-	-	K

FIGURE 2. Peptide analogues of m88-103.

At least two substitutions were made for each position, one of which was selected in order to minimize changes in the charge, hydrophobicity, or size of the analogues relative to that of the native peptide. The other was to a hydrophobic residue with

a conformational preference for the native α-helical conformation. For those sequence positions that were not already alanine, the change was made to alanine, A. In Figs. 1 and 2, and in the text, we will refer to the peptides by denoting the substituted residue, for example, the peptide K104R has the native sequence 88–104 with a substitution of arginine for lysine at position 104.

The peptides were tested in vitro for their ability to stimulate the three T cell hybridomas in the presence of various APC. A representative dose-response curve for the hybridoma 22.D11/h with p88–104, presented by B10.A APC, is shown in Fig. 3 *a*. The half-maximum value of the native peptide was determined from the dose-response curve as the concentration of the antigen required to stimulate 50% of the maximal response of 20,850 cpm, and the dose of each peptide to stimulate 11,000 cpm was obtained. The values obtained for analogues were normalized to that of the native peptide p88–104. The data for the response of T cell hybridoma 22.D11/h to pcyt *c* analogues on B10.A or B10.A(5R) APC are summarized in Table II. Most analogues lost their antigenic potency, even at the higher concentration of 100 μM/well (20 times the concentration of the native peptide required for maximal stimulation), except for the analogues with substitutions at positions 98 and 100 in the presence of B10.A APC and at positions 98, 100, and 102 in the presence of B10.A (5R) APC. Some of the nonstimulatory analogues had slight stimulatory activity at 100 μM/well with half-maximum values that can be extrapolated to be in the range of 100–500 (data not shown). With the exception of these slightly stimula-

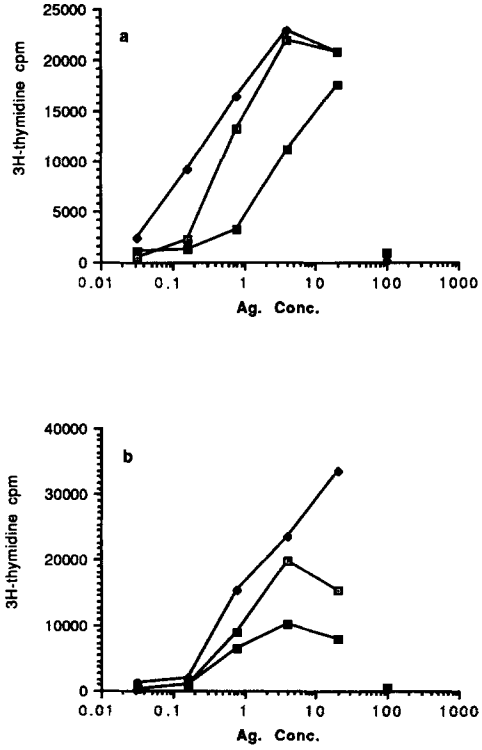

FIGURE 3. Competition assays between nonstimulatory analogues and native peptide for presentation to 22.D11/h T cells by B10.A APC. (*a*) The nonstimulatory analogues were added to the culture at the same time as the native peptide p88–104. (*b*) The native peptide was incubated with the APC for 5 h before the addition of the analogue and the 22.D11/h T cells. 88–104 (⊡) half-maximum value, 0.6 μM (*a*); 1.0 μM (*b*); 88–104 + Y97A (◆) half-maximum value, 0.24 μM (*a*); 0.41 μM (*b*); 88–104 + Y102S (■) half-maximum value, 4.2 μM (*a*); 4.0 μM (*b*).

tory peptides, all peptide analogues that were nonstimulatory at 100 μM/well were used in competition with the native peptide 88–104 for presentation to the T cell hybridomas, as described in Materials and Methods.

An example of the effect on the dose-response curve of the native peptide 88–104 on adding either of the two peptide analogues, T102S and Y97A, is shown in Fig. 3 *a*. In this experiment, the native peptide was titrated from 20 μM/well down to 0.032 μM/well in fivefold dilutions. The analogue, Y97A or T102S, was then added to each row and the control wells in the absence of the native peptide. Irradiated spleen cells were added and the plates were incubated for 2 h before 22.D11/h T hybridoma cells were added. Fig. 3 *a* shows that the analogue T102S reduced the recognition of p88–104 (B in Table II) while the analogue Y97A enhanced the response to p88–104 (E in Table II). Some of the peptide analogues had no effect on

TABLE II

Relative Concentration of pcyt c Peptide Required for Stimulation of Phenotype IV T Cell Hybridoma 22.D11/h

	B10.A APC		B10.A(5R) APC	
Peptide	Relative concentration	B/E/N*	Relative concentration	B/E/N
p88–104	1‡		1‡	
K104R	ND		7.8	
A103K, K104A	>100	B	>100	N
K104A	>100	B	>100	N
A103K	>100	B	>100	N
A103I	>100	B	>100	N
T102A	>100	-	0.2	
T102S	>100	B	48	
A101I	>100	B	>100	-
A101Y	>100	B	>100	-
Q100N	13		0.42	
Q100A	2		0.27	
K99A	>100	B	>100	-
K99R	>100	B	>100	-
L98F	1.5		0.38	
L98A	20		0.84	
Y97A	>100	E	>100	N
Y97I	>100	N	>100	N
A96Y	>100	-	>100	N
A96I	>100	N	>100	N
I95A	>100	-	>100	-
I95Y	>100	-	>100	-

* The behavior of the peptides in competition with p88–104. B, blocking; E, enhancement; N, no effect; -, slight stimulatory activity, therefore not tested in competition assay.

‡ Maximum response of: p88–104 on B10.A, 20,000–40,000 cpm; p88–104 on B10.A(5R), 10,000–20,000 cpm. Half-maximum value of: p88–104 on B10.A, 0.1–1 μM; p88–104 on B10.A(5R), 2–8 μM.

the recognition of the native peptide and are denoted as N in Table II. The weakly stimulatory peptides, which were omitted from the competition experiments, are denoted in Table II by a dash.

In other experiments, APC were preincubated with the native peptide (at a range of dilutions) for 5 h before the addition of the nonstimulatory analogue (100 μM/well) and the T cells. However, the later addition did not appear to change either the blocking or enhancing ability of a peptide analogue. Fig. 3 *b* shows competition assays for T102S and Y97A performed in this way. We could not demonstrate any significant blocking or enhancing in the competition experiments with nonstimulatory analogues performed using B10.A(5R) APC and the T cell hybridoma 22.D11/h. We believe this may be because of the weaker response of 22.D11/h to p88–104 on B10.A(5R) cells.

Different Residues within 95–104 Serve as Agretopes or Epitopes for the Three Different Hybridomas. It has previously been shown that a residue may function as an epitope defined as a T cell binding site, or an agretope, an MHC binding site, or both simultaneously (12, 22–24). In our assays, we define an epitope as a residue, which when substituted, renders the analogue nonstimulatory but still capable of blocking or enhancing the response of the native peptide in a competition assay. Thus, the analogue can compete at the level of MHC binding (agretope) and retains this function. Applying this definition to the data shown in Table II, it can be seen that examples of residues that function as epitopes in the 22.D11/h response to p88–104 on B10.A APC are residues 104, 103, 102, 101, 99, and 97.

An agretope is defined as a residue that shows a significant shift in relative concentration from one MHC (B10.A:$E^k_\beta E^k_\alpha$) to another (B10.A [5R]:$E^b_\beta E^k_\alpha$ or B10.S[9R]: $E^s_\beta E^k_\alpha$) when presented to the same T cell hybridoma (7). For example, the data in Table II show that substituting an alanine for a threonine at residue 102 results in a change in the half-maximum value of the peptide, T102A, from >100, using B10.A APC, to 0.2 on B10.A(5R) APC. Thus, from Table II it can be seen that residues 98, 100, and 102 are agretopes for T cell hybridoma 22.D11/h. A schematic model of p88–104, as seen by 22.D11/h on B10.A and B10.A(5R) APC, is depicted in Fig. 4. It should be noted that a number of residue changes that abrogate recognition by the T cell cannot be assigned either an epitopic or agretopic function based on our criteria, and these are shown as U (unassigned) in Fig. 4.

Similar experiments were carried out with T cell hybridomas 2H10.a and 2B4.49. 2H10.a responds to pcyt *c* on B10.A APC ($E^k_\beta E^k_\alpha$) and B10.S (9R) APC ($E^s_\beta E^k_\alpha$), and also responds to mcyt *c* on B10.A APC. The results, shown in Tables III and IV and schematically presented in Fig. 4, define which residues appear to function as epitopes and agretopes for T cell hybridoma 2H10.a when p88–104 analogues are presented on B10.A and B10.S(9R) APC and when m88–103 analogues are presented on B10.A APC. The residues 97 and 102 on m88–103, and 97, 101, and 103 on p88–104, were recognized as epitopes (Fig. 4). Since the response of 2H10.a to mcyt *c* is restricted to $E^k_\beta E^k_\alpha$ only, it was not possible to define agretopes on mcyt *c* using our criteria, but for pcyt *c*, agretopic residues are 95, 97, 98, and 101.

T cell hybridoma 2B4.49, on the other hand, responds to pcyt *c* on B10.A APC and mcyt *c* on B10.A and B10.A(5R) APC. Tables V and VI list the data of direct stimulation assays and competition assays. It is interesting to note that no blocking was observed with any of the nonstimulatory analogues, but some of them enhanced

Pigeon cyt c as recognized by 22.D11

95	96	97	98	99	100	101	102	103	104
U	U	E	A	E	A	E	E/A	E	E

Pigeon cyt c as recognized by 2H10.a

95	96	97	98	99	100	101	102	103	104
A	U	E/A	A	U	U	E/A	U	E	U

Moth cyt c as recognized by 2H10.a

96	97	98	99	100	101	102	103
U	E	U	U	U	U	E	U

Pigeon cyt c as recognized by 2B4.49

95	96	97	98	99	100	101	102	103	104
U	U	E	-	E	U	E	U	-	E

Moth cyt c as recognized by 2B4.49

96	97	98	99	100	101	102	103
-	U	A	U	-	E	U	E/A

FIGURE 4. A summary of epitope and agretope assignments for the pcyt *c* sequence 95–104 and the mcyt *c* sequence 95–103 for T cell hybridomas 22.D11, 2H10.a, and 2B4.49. E, epitope; A, agretope; U, insufficient data to assign a function to this residue; and -, no affect on T cell recognition.

the response of the native peptide. We have classified those residues that on substitution resulted in such enhancement as epitopes. From Tables V and VI, and the summary in Fig. 4, the data show that the 2B4.49 TCR interacts with residues 101 and 103 in m88–103 and 104, 101, 99, and 97 on the pigeon peptide. Agretopes 103 and 98 on the mcyt *c* peptide are defined on the basis of the shift in half-maximum value between $E^k_\beta E^k_\alpha$ and $E^b_\beta E^k_\alpha$APC.

Fig. 4 summarizes all the data shown in Tables II to VI, to facilitate a comparison of the way in which sequences p95–104 and m95–103 are recognized by the three different hybridomas with respect to the residues assigned as epitopes and agretopes.

Discussion

In this study we have analyzed peptide binding patterns of the pcyt *c* and mcyt *c* peptide antigens when in complex with different MHC-TCR combinations. These antigens are restricted to MHC I-E^k_β and to its congenic variants I-E^b_β and I-E^s_β. Upon injection into B10.A mice, they elicit a limited but diverse T cell repertoire that uses α and β genes from a common pool of genes (25).

Pcyt *c*–specific T cells have been classified into five phenotypes according to their fine specificities (9). In the experiments described here, T cell hybridomas, 22.D11/h (type IV), 2H10.a (type IIIa), and 2B4.49 (type I) were used as responder cells (Table I). To determine the epitopic residues, competition assays were set up in which the native peptide and competing peptide were added together to the APC and the T cells. When the APC were preincubated with the native peptide for 5 h, washed three times in complete medium before the addition of the competing peptide, and then assayed with the T cells, a very similar pattern of blocking and enhancement

TABLE III

Relative Concentration of pcyt c Peptide Required for Stimulation of Phenotype IIIa T Cell Hybridoma 2H10.a

Peptide	B10.A APC		B10.S(9R) APC	
	Relative concentration	B/E/N*	Relative concentration	B/E/N
p88-104	1‡		1‡	
K104R	1.6		1	
A103K, K104A	1.6		0.4	
K104A	>100	-	>100	N
A103K	0.3		0.9	
A103I	>100	B	>100	B
T102A	>100	N	>100	N
T102S	>100	N	>100	
A101I	>100	-	>100	N
A101Y	6.6		>100	B
Q100N	>100	-	>100	N
Q100A	0.6		0.42	
K99A	>100	-	>100	-
K99R	>100	-	>100	-
L98F	1		>100	ND
L98A	>100	N	0.225	
Y97A	>100	E	>100	N
Y97I	>100	N	0.3	
A96Y	>100	N	>100	N
A96I	>100	-	>100	N
I95A	>100	N	>100	N
I95Y	>100	N	0.32	

* The behavior of the peptides in competition with p88-104. B, blocking; E, enhancement; N, no effect; -, slight stimulatory activity, therefore not tested in competition assay.

‡ Maximum response of: p88-104 on B10.A, 18,000-30,000 cpm; p88-104 on B10.S(9R), 10,000-30,000 cpm. Half-maximum value of: p88-104 on B10.A, 0.5-5 μM; p88-104 on B10.S(9R), 0.25-8 μM.

was observed (compare Fig. 1, *a* and *b*). This result suggests that the analogues and the native peptide have a similar affinity for the MHC molecule. In these competition experiments, it was not necessary to substitute peptides of weak stimulatory activity for the native peptide, or to use lower concentrations of the native peptide, in order to detect blocking by the nonstimulatory analogues, as was reported in other studies (26, 27).

We saw two effects from the addition of nonstimulatory analogues in the competition assays. In most cases a diminution in the response of the native peptide was observed, which indicates that the nonstimulatory analogue competes with the native peptide at the MHC-binding level, hence, the agretope is intact and functional. Alternatively, the presence of nonstimulatory peptides sometimes resulted in an en-

TABLE IV

Relative Concentration of mcyt c Peptide Required for Stimulation of Phenotype IIIa T Cell Hybridoma 2H10.a

	B10.A APC	
Peptide	Relative concentration	B/E/N*
m88-103	1‡	
K103A	>100	-
K103R	>100	-
T102V	>100	B
T102A	>100	ND
A101I	>100	ND
A101Y	>100	ND
Q100N	>100	-
Q100A	>100	-
K99A	>100	N
K99R	>100	N
L98F	7.8	
L98A	ND	
Y97A	>100	-
Y97I	>100	B
A96Y	22.8	
A96I	25	

* The behavior of the peptides in competition with m88-103. B, blocking; E, enhancement; N, no effect; -, slight stimulatory activity, therefore not used in competition assay.

‡ Maximum response of m88-103 on B10.A, 20,000-25,000 cpm; half-maximum value of m88-103 on B10.A, 0.1-5 μM.

hanced response of the native peptide. This observation, that nonstimulatory peptides can enhance the recognition of the native sequence, is very surprising. The mechanism by which enhancement can occur is under current investigation, but one may speculate that the analogue may be involved in the reorientation of the native peptide to facilitate a better contact with the TCR, or alternatively, that the MHC groove can accommodate two peptides in an extended form that act in synergy to stimulate T cells. Either explanation argues against a single peptide binding the MHC molecule, as proposed by others (2). In this study we have classified a residue change that renders a peptide nonstimulatory, but has an enhancing effect in competition assays with the native peptide, as an epitope, since the substitution has clearly not affected the ability of the peptide to bind to the MHC.

For the T cell hybridoma 22.D11/h, the residues 97, 99, 101, 102, 103, and 104 were found to be epitopes, which implies that a TCR of this specificity sees the pcyt *c* peptide presented in a linear fashion. The epitopes and the agretopes of pcyt *c* for 22.D11/h are shown in Fig. 4, in which residues 95 and 96 are unassigned (U) because, although substitutions at these positions affected recognition, they could not be classified by our criteria. From the above results, it is evident that the TCR of T cell hybridoma 22.D11/h interacts with the last four residues of the pcyt *c* pep-

TABLE V

Relative Concentration of pcyt c Peptide Required for Stimulation of Phenotype I T Cell Hybridoma 2B4.49

	B10.A APC	
Peptide	Relative concentration	B/E/N*
p88-104	1‡	
K104R	0.4	
A103K, K104A	ND	
K104A	>100	E
A103K	0.27	
A103I	11	
T102A	>100	N
T102S	67	
A101I	>100	E
A101Y	29	
Q100N	>100	-
Q100A	2.5	
K99A	11.7	
K99R	>100	E
L98F	0.27	
L98A	2.5	
Y97A	>100	E
Y97I	>100	E
A96Y	>100	-
A96I	>100	N
I95A	>100	N
I95Y	ND	

* The behavior of the peptides in competition with p88-104. B, blocking; E, enhancement; N, no effect; -, slight stimulatory activity, therefore not tested in competition assay.

‡ Maximum response of p88-104 on B10.A, 25,000-60,000 cpm; half-maximum value of p88-104 on B10.A, 0.2-2 μM.

tide. The epitopes at positions 103 and 104 may play an important role in this specificity since the moth analogue, which differs from the pigeon peptide at positions 103 and 104, is not recognized by this T cell hybridoma (8, 9).

Similar experiments with pcyt *c* peptides on T cell hybridomas 2H10.a and 2B4.49 are listed in Tables III and V. As was the case for 22.D11/h (Table II), it can be seen that a large number of the analogues were nonstimulatory. With 2H10.a cells, which respond to p88-104 on B10.A and B10.S(9R) APC, most of the analogues were nonstimulatory at 100 μM/ml. The agretopes were found to be in positions 97, 98, and 101, the epitopes at positions 97, 99, 101, and 103, and the residues 96, 99, 100, 102, and 104 were not assigned to either classification (Table III and Fig. 4). Hybridoma 2B4 does not respond to p88-104 on either B10.A(5R) or B10.S(9R) APC, therefore, it was not possible to define the agretopes for the pigeon peptide using our criteria. The epitopes were found at positions 97, 99, 101, and 104 (Table V and Fig. 4).

TABLE VI

Relative Concentration of mcyt c Peptide Analogues Required for Stimulation of T Cell Hybridoma 2B4.49

	B10.A APC		B10.A(5R) APC	
Peptide	Relative concentration	B/E/N*	Relative concentration	B/E/N
m88-103	1‡		1	
K103A	3.94		>100	E
A103R	51.5		>100	E
T102V	>100	-	>100	-
T102A	23		17	
A101I	>100	E	>100	-
A101Y	98		>100	-
Q100N	9.5		26.3	
Q100A	3.4		1.05	
K99A	>100	-	>100	-
K99R	84		28	
L98F	1.32		3.6	
L98A	5.4		>100	-
Y97A	>100	-	>100	-
Y97I	84		>100	-
A96Y	0.2		0.7	
A96I	1.03		3.6	

* The behavior of the peptides in competition with m88-103. B, blocking; E, enhancement; N, no effect; -, slight stimulatory activity, therefore not tested in competition assay.

‡ Maximum response of: m88-103 on B10.A, 15,000-60,000 cpm; m88-103 on B10.A(5R), 15,000-35,000 cpm. Half-maximum value of: m88-103 on B10.A, 0.1-1 μM; m88-103 on B10.A(5R), 2-8 μM.

The failure of T cells with the type I phenotype, such as 2B4, to recognize pcyt *c* when presented on B10.A(5R) cells has been attributed to the reduced ability of p81-104 to interact with the I-E^b_β molecule, as compared with I-E^k_β. We have previously suggested (8) that the ability of 22.D11 to be stimulated by p81-104 on I-E^b_β expressing APC argues against this notion. Our finding, in these studies, that different residues appear to be used as agretopes and epitopes by the three different phenotypes, supports our previous hypothesis (8) that the interaction of p81-104 with I-E^b_β (but not I-E^k_β) masks the recognition of residues involved in TCR recognition of T cells of the type I phenotype but not for cells of the type IV phenotype.

Similar substitutions in m88-103 generated a set of peptides, as listed in Fig. 2, and were analyzed in a similar manner on 2H10 and 2B4; 22.D11/h does not recognize this peptide. The results of these experiments with 2H10.a are shown in Table IV and Fig. 4, and those with 2B4.49 with B10.A or B10.S(9R) APC are shown in Table VI and Fig. 4. The epitopes of m88-103 seen by the two T cell hybridomas are not the same. In addition, a particular TCR (2H10.a or 2B4.49) does not appear to recognize the moth and pigeon peptides in the same configuration, as there is little or no overlap of epitopes in the two peptides (Fig. 4).

These results show, first, that every residue between 95 and 104/103 contributes to the antigenicity of the peptide for hybridomas 22.D11/h and 2H10.a, and that there were only two residues (99 and 103 in p88–104 and 96 and 100 in m88–103) in which substitutions had no effect on the recognition of 2B4.49. These residues were not the same neutral residues as described by Fox et al. (12) for other type I T cell specificities. In this study, in which a series of analogues between 98 and 104 were analyzed using T cell clones A.E7 and F1.A2, residues 98 and 104 were found to be neutral, residues 99–102 were defined as epitopic, and 100–103 were defined as agretopic. The contrast between these findings and our own emphasize the diversity of recognition of a single peptide, even within T cell clones of similar specificity. A comparison of the fine specificities of T cell clones within the phenotypic classifications of I, III, IV, and V confirms this finding (Sorger, S. B., Y. Paterson, P. J. Fink, and S. M. Hedrick, manuscript submitted for publication).

A second implication of our findings is that the residues of any given peptide that are used to interact with the MHC molecule are not fixed for every MHC-TCR interaction; that is, the same peptide may bind one MHC molecule through different sets of agretopic residues, thus exposing different residues to different TCR. Thus, the distribution of epitopes for both the moth and pigeon peptide also varies for each type of T cell hybridoma. A similar conclusion was reached by Plachov et al. (28) in studies on the fine specificity of the I-$A^{b/k}$-restricted T cell response to pig insulin. These data emphasize the ternary nature of the MHC-TCR-ligand interaction and clearly rule out a static model of T cell recognition, which assigns certain invariant agretopic residues to the peptide by which it interacts with the MHC molecule independently of the TCR. Thus, our assignments of those residues that interact with MHC or TCR are valid only for the particular trimolecular complex from which they were derived.

Our findings are consistent with a requirement for the TCR in stabilizing the complex, as has been suggested by others (29, 30), and are in contrast with the proposal that a single peptide associates with the Ia molecule in a specific conformation (31) that generates TCR diversity by overlapping epitopes being present on this single molecular species. Indeed it has been suggested that T cell determinants are recognized predominantly as amphipathic α-helices, such that the residues contacting the TCR lie on the hydrophilic face of the helix, while the agretopic residues are arranged on the hydrophobic face (32, 33). If we arrange the assigned agretopes and epitopes of p95–104 (for the hybridomas 22.D11/h and 2H10.a) and m95–103 (for 2B4.49) in a helical configuration (Fig. 5), there is no segregation of agretopes and epitopes on opposite faces of the helix of p95–104 for 22.D11/h and 2H10.a or m95–103 for 2B4.49. This strongly suggests that these T cell determinants are not recognized by the three TCRs in a helical conformation. These findings are in agreement with other studies from our laboratory (16, 34), which show that increasing the helical propensity of the T cell determinant 95–104 by adding non-native extensions at the NH_2 terminus does not result in improved recognition of the peptide by the T cell hybridomas used in this study.

The promiscuity of peptide binding to MHC, in different conformations, may explain the diversity in the fine specificity of the pcyt *c*– and mcyt *c*–specific T cell clones. The expression of these clones varies quantitatively, that is, the phenotype I is the most commonly expressed T cell type. It is not clear whether there is a preferen-

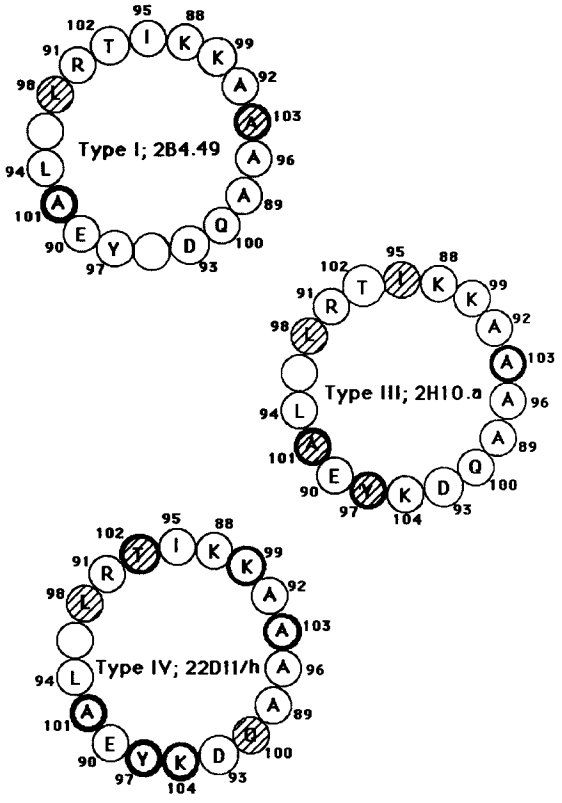

FIGURE 5. Residue assignments summarized in Fig. 4 plotted as a helical wheel to illustrate the position of agretopes and epitopes in the peptide if it adopted an α-helical conformation. The assignments for p88–014 are shown for 22.D11/h and 2H10.a. For 2B4.49, for which agretope assignments can only be made for m88–103, these are shown.

tial selection of a particular Vβ at the thymic level or whether the common expression of a particular TCR Vβ is a consequence of its preferential expansion due to a particularly stable configuration of the TCR-MHC-peptide complex. The latter explanation seems more plausible if a peptide has the flexibility of binding in a variety of ways, of which some are more stable than others.

Summary

The interaction of TCR, antigen, and MHC complex has been analyzed using synthetic peptide antigens and a series of single amino acid-substituted analogues. Two similar antigens, mouse cytochrome *c* (mcyt *c*) and pigeon cytochrome *c* (pcyt *c*), elicit T cell responses in strains of mice bearing MHC class II $E^k_\beta E^k_\alpha$ (B10.A), $E^b_\beta E^k_\alpha$[B10.A(5R)], and $E^s_\beta E^k_\alpha$[B10.S(9R)]. The immunogenic regions of these antigens are located in the peptide sequence p88–104 for pcyt *c* and m88–103 for mcyt *c*. The limited T cell repertoire for these antigens is comprised of four groups of T cell phenotypes that have very few differences in their TCR gene make up. In this paper, we examine the diversity in their fine specificity for each of the antigens, m88–103 and p88–104, complexed with each of the I-E^k haplotypes. Epitopes, i.e, residues that interact with the TCR, and agretopes, i.e., residues in the MHC-binding site, were assigned for the two peptide antigens in the presence of APC bearing $E_\beta{}^k E^k_\alpha$, $E^b_\beta E^k_\alpha$, or $E^b_\beta E^k_\alpha$ using T cell hybridomas of the phenotypes I, IIIa, and IV. From

our results, we conclude that first, the substitution of any residue between 95 and 104 of the cytochrome *c* peptide changed the antigenic potency of the peptide for at least one of the hybridomas. Second, each T cell type has a different recognition pattern of epitopes and agretopes for a particular antigen-MHC complex, thus, ruling out a static model of T cell recognition, which assigns certain, invariant agretopic residues to the peptide by which it interacts with the MHC molecule independently of the TCR. Third, the same T cell hybridoma responded to the antigens differently when presented on various MHC molecules, implying that overall changes in the MHC groove, as displayed by the three haplotypes, may affect the efficiency in binding the peptide. Fourth, since most of the residues are used as epitopes by at least one of the T cell specificities, the peptide appears to be recognized in a different conformation by each T cell hybridoma phenotype; and, finally, the epitopic and agretopic residues do not segregate, for any one of the T cell specificities, in such a way that suggests they are recognized in a helical conformation. In summary, our results suggest that a single peptide may generate diversity in the T cell response by virtue of its conformational flexibility within the TCR-MHC-antigen complex.

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