

Specific and General HLA-DR Binding Motifs: Comparison of Algorithms

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ABSTRACT: Using panels of peptides well characterized for their ability to bind to HLA DR1, DRB1*1101, or DRB1*0401 molecules, algorithms were deduced to predict binding to these molecules. These algorithms consist of blocks of 8 amino acids containing an amino acid anchor (Tyr, Phe, Trp, Leu, Ile, or Val) at position i and different amino acid combinations at positions $i+2$ to $i+7$ depending on the class II molecule. The sensitivity (% of correctly predicted binder peptides) and specificity (% of correctly predicted non-binder peptides) of these algorithms, were tested against different independent panels of peptides and compared to other algorithms reported in the literature. Similarly, using a panel of 232 peptides able to bind to one or more HLA molecules as well as 43 non-binder peptides, we deduced a general motif for the prediction of binding to HLA-DR molecules. The sensitivity and specificity of this general motif was dependent on the threshold score used for the predictions. For a score of 0.1, the sensitivity and specificity

were 84.7% and 69.8%, respectively. This motif was validated against several panels of binder and non-binder peptides reported in the literature, as well as against 35, 15-mer peptides from hepatitis C virus core protein, that were synthesized and tested in a binding assay against a panel of 19 HLA-DR molecules. The sensitivities and specificities against these panels of peptides were similar to those attained against the panels used to deduce the algorithm. These results show that comparison of binder and non-binder peptides, as well as correcting for the relative abundance of amino acids in proteins, is a useful approach to deduce performing algorithms to predict binding to HLA molecules. *Human Immunology* 61, 266–278 (2000). © American Society for Histocompatibility and Immunogenetics, 2000. Published by Elsevier Science Inc.

KEYWORDS: T cell determinant; class II molecules; algorithm; MHC; binding motif; prediction of binding

ABBREVIATIONS

APC antigen presenting cell
MHC major histocompatibility complex

HCV hepatitis C virus
TDh T-helper cell determinant

INTRODUCTION

Determinants recognized by T-helper cells (TD_h) are peptides of 8 to 23 amino acids (commonly 14 to 15 in average) that are able to bind to MHC class II molecules. TD_h originate generally in endocytic cell compartments after processing of foreign protein antigens, that have

entered the antigen presenting cell (APC) following immunization with an antigen or after infection, although peptides derived from self proteins also bind to class II molecules. Presentation of the MHC class II-peptide complex by an APC, followed by its recognition by the T cell receptor, leads to T cell activation. See [1] for a thorough review on these findings.

It has been shown [2] that TD_h peptides bind to a single site in a groove of the MHC class II molecules. Also, the association pattern of peptides to different allele variants of murine Ia is a reflection of the MHC restriction of the immune response [3, 4]. Several workers have attempted to unravel the relevant struc-

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tural requirements of TD_h peptides to interact with murine [5–7] or human [8–14] class II molecules. The X-ray structure of a class II molecule [15] has confirmed that the groove is occupied by a single peptide, and also, that its structure is similar to the one found for class I molecules [16]. However, the binding site of class I molecules accommodates peptides of a length of 8 to 10 amino acids [17], whereas peptides eluted from class II molecules may vary between 8 to 23 amino acids [18].

Because TD_h play a fundamental role in the induction of humoral [19] and cytotoxic responses [20–24] their identification is of paramount importance. Several groups have published algorithms for the prediction of TD_h from proteins. These algorithms are based on several principles: (i) amino acid sequence patterns [25]; (ii) tendency of peptides to form amphipathic alpha helices [26]; (iii) tendency of peptides to form strip-of-helices [27]; (iv) amino acid sequence analogies with known T cell epitopes [7, 28]; (v) peptides eluted from M13 phages display libraries [29, 30]; (vi) elution of peptides from HLA-DR molecules [31]; and (vii) prediction of peptide binding by comparison with a model peptide [32]. However, as discussed in the present publication, these algorithms have a wide range of sensitivities and specificities.

TD_h peptides have an hydrophobic amino acid residue near the N-terminus that plays the role of anchor for binding to class II molecules [14, 33]. The shorter TD_h peptides are usually 9 amino acids long [34], an observation that has been confirmed by others [29, 30] when eluting peptides from DR1, HLA DRB1*1101, and HLA DRB1*0401 molecules expressed by filamentous M13 phages. In all three cases the eluted peptides were 9 amino acids long. Most of these peptides contained an hydrophobic anchor amino acid [13] (Tyr, Phe, Trp, Leu, Ile, or Val), at the N-terminus or one residue away from the N-terminus. Since the anchor residue could be situated at position 2, the minimal binding block might have a length of 8 amino acids. It occurred to us that by comparing blocks of 8 amino acids, in groups of binder and non-binder peptides to different HLA-DR molecules, algorithms could be developed for the prediction of peptide binding to these molecules. Similarly, by comparing blocks of peptides that were able to bind to one or more HLA-DR molecules (DR1, DR2, DR5, DR52a) with 43 blocks of peptides that were unable to bind to these four HLA-DR restrictions [33], we deduced an algorithm for the prediction of peptide binding to either of these molecules. These developments are discussed in detail below and are the main aim of the present publication.

MATERIALS AND METHODS

Peptide Synthesis

Thirty-five 15-mer peptides from hepatitis C virus core protein genotype 1b [35] were synthesized manually by the solid phase method of Merrifield [36] using the Fmoc alternative [37] and a multiple solid phase peptide synthesizer [38]. Peptide HA(306–320) (CPKYVKQNTLKLATG) from Influenza A/Texas/77 virus hemagglutinin was synthesized manually, and biotinylated (while still attached to the resin) with an excess of N-hydroxy-succinimide-conjugated biotin (NH-LC-biotin) (Pierce). Completion of biotinylation was assessed by the ninhydrin test of Kaiser [39]. All peptides were at least 80% pure as assessed by HPLC.

Cells and mAb

EBV-transformed B lymphoblastoid cell lines (EBV-BLCL) BGE, TISI, FPAF, and MOU were obtained from the European Collection of Animal Cell Cultures (ECACC, PHLS, Salisbury, UK). The L243 anti-DR and W6/32 anti-class I hybridomas were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The remaining EBV-BLCL (issued from the Tenth Histocompatibility Workshop), the 33.1 anti-DQ and the B7/21 anti-DP mAb were provided by Dr. Ghislaine Sterkers. The RM3 cell line (DR-, DQ-, DP-) derived from the human Burkitt lymphoma cell line Raji was provided by Dr. Bernard Bénichou. EBV-BLCL and the RM3 cell line were grown in RPMI 1640 (Whittaker Bioproducts) supplemented with 10% FCS (INC Flow), penicillin and streptomycin. The 19 HLA-DR homozygous EBV-BLCL used in the binding assay were: JESTOM (DRB1*0101), MZ070782 (DRB1*0102), MGAR (DRB1*1501/ DRB5*0101), BGE (DRB1*1502/ DRB5*0102), KAS011 (DRB1*1601/ DRB5*0201), VAVY (DRB1*0301/ DRB3*0101), RSH (DRB1*0302/ DRB3*0101), BOLETH (DRB1*0401/ DRB4*0101), YAR (DRB1*0402/ DRB4*0101), SWEIG (DRB1*1101/ DRB3*0202), JVM (DRB1*1102/ DRB3*0202), TISI (DRB1*1103/ DRB3*0202), FPAF (DRB1*1104/ DRB3*0202), CB6B (DRB1*1301/ DRB3*0202), SLE005 (DRB1*1302/ DRB3*0301), AMALA (DRB1*1402/ DRB3*0101), DRB1*0101MOU (DRB1*0701/ DRB4*0101), OLGA (DRB1*0802), DKB (DRB1*0901/ DRB4*0101).

Peptide Binding Assays

Binding assays were performed as previously described [9, 14]. Briefly, 3.5×10^5 EBV-BLCL were co-incubated during 4 hours with biotinylated HA(306–320) (10 μ M) and unbiotinylated HA(306–320) (150 μ M) or with biotinylated HA(306–320) (10 μ M), and the peptide to be tested (150 μ M). Cells were washed twice at

4°C with 2 ml of PBS/0.1% BSA, re-suspended in 5 µg/ml of streptavidin-fluorescein conjugate (Pierce), and incubated at 4°C with 2 ml of PBS/0.1% BSA. Following re-suspension in 300 µl PBS/0.1% BSA, the cell surface fluorescence was measured by flow cytometry on a FACScan analyzer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). The mean fluorescence of 5000 stained cells was determined. Dead cells were excluded from the analysis by staining with propidium iodide (1 µg/ml). Background was measured as above but in the absence of biotinylated peptide. This value was subtracted from all measurements. All assays were performed by triplicate. To compensate differences in HLA-DR expression between EBV-BLCL, the fluorescence obtained with biotinylated peptide and fluoresceinated streptavidin, was divided by the fluorescence obtained after staining the corresponding EBV-BLCL with an excess of FITC-conjugated L243 mAb (Becton Dickinson). Specificity was demonstrated by inhibition of binding using anti-DR, anti-DQ, anti-DP, and anti-class I mAb (kindly provided by Dr. Ghislaine Sterkers) by competition with unbiotinylated HA(306–320) (data not shown).

The relative inhibitory capacity (RI%) of peptides from HCV core protein was calculated according to the formula:

$$RI\% = 100 \times (\text{signal inhibition with peptide tested}) / (\text{signal inhibition with unbiotinylated HA(306–320)}).$$

The fluorescence intensities of triplicate samples were usually within 5% of the mean and always within 10%.

Statistical Methods

One sample Kolmogorov-Smirnov (Lilliefors) test was used to assess normality. For variables not normally distributed, Mann-Whitney's *U* was used. For variables normally distributed, Student's *t* test for independent samples was used. One sample proportion *Z* test was used to compare amino acid relative frequencies observed to the relative frequencies expected. χ^2 test was used to measure the association between binary (\pm) variables.

Average Amino Acid Frequencies

Average amino acid frequencies, used throughout this article, were calculated from the amino acid composition of 23406 protein sequences (8224555 amino acids) from the Swissprot data base. These frequencies were the following: Ala (7.8%), Cys (1.8%), Asp (5.3%), Glu (6.3%), Phe (4.0%), Gly (7.3%), His (2.3%), Ile (5.5%), Lys (5.9%), Leu (9.1%), Met (2.3%), Asn (4.4%), Pro (5.1%), Gln (4.1%), Arg (5.2%), Ser (7.0%), Thr (5.8%), Val (6.6%), Trp (1.3%), Tyr (3.2%).

Relative Abundance of Amino Acids in Binder and Non-Binder Peptides to Different HLA-DR Molecules

By comparing the amino acid sequences from blocks of 8 amino acids in binder and non-binder peptides to different HLA-DR molecules, containing an anchor residue at their *N*-terminal position (Tyr, Phe, Trp, Leu, Ile, or Val), algorithms were deduced to predict peptide binding to these molecules. Thus, 77 blocks of 8 amino acids encompassed by 43 peptides, that according to O'Sullivan et al. [33], were unable to bind to DR1, DR2, DR5, or DR52a, were compared with similar blocks from 60, 52, and 52 binder peptides, eluted from DR1, DRB1*1101, and DRB1*0401 molecules, respectively, expressed by filamentous M13 phages [29, 30]. For the case of DR1, besides the 43 peptides that were unable to bind to any of the four HLA-DR tested, another 28 non-binder peptides to DR1 [33] were considered.

Comparisons between binder and non-binder blocks were done as follows: The percentage of abundance (relative frequency) of the 20 natural amino acids at positions (*i*+1 to *i*+7) of blocks of 8 amino acids containing an anchor residue at position *i*, was calculated both in the population of binder and non-binder peptides (data not shown). The frequency of abundance of these amino acids in 23406 proteins from the Swissprot data base was also calculated. To assess that differences in relative frequencies of each amino acid at every position of the block, in the group of binders as well as in non-binders, respect to the reference frequencies (those obtained from the Swissprot data base) were not due to chance alone, one sample proportion *Z* values for each amino acid at each position in binder and non-binder peptides were calculated. The frequencies of every amino acid at positions *i*+1 to *i*+7 in the blocks of binders and non-binders were then divided by the frequency of the corresponding amino acid in the Swissprot data base. These relative frequencies of abundance thus calculated, in conjunction with the *Z* values, were used to define amino acids having enhancing, deleterious or neutral effect on binding of peptides to HLA-DR molecules. Thus, an amino acid was considered to have an enhancing effect, when the ratio between its relative frequency in binders and non-binders was equal or greater than 1.75 (75% greater than the observed value in non-binders) and also, that its *Z* value was greater than 1.28 in binders but not in non-binders. Similarly, an amino acid was considered deleterious when, the ratio between its relative frequency in non-binders and binders was equal or greater than 1.75 and its *Z* value was lower than -1.28 in binder but not in non-binder blocks. The *Z* values of 1.28 and of -1.28 are associated with a *p* < 0.1, which was considered as significant. Those amino acids that did not be-

TABLE 1 Ratios of relative frequency of abundance of amino acids of binding relevance^a for the prediction of peptide binding to DR1, DRB1*0401 and DRB1*1101

DR1							
Y, F, W	R = 3.06		M = 13.85				H = 4.30
L, I, V	H = 2.87	Q = 3.25	L = 2.51	M = 10.51			M = 1.91
	Q = 1.79	M = 1.91	Q = 2.48	Q = 1.91	A = 2.39	P = 2.05	Q = 1.91
i	i + 1	i + 2	i + 3	i + 4	i + 5	i + 6	i + 7
	S = -2.09	E = -2.30	I = -1.83	D = -2.36	D = -2.44	D = -1.88	D = -2.79
	E = -5.23	D = -2.62	D = -3.14		I = -4.71	D = -2.62	S = -3.66
	P = -10.47	I = -3.66	S = -3.14			I = -4.71	F = -5.23
		F = -4.19	R = -3.49				
DRB1*0401							
			M = 5.55				
Y, F, W	R = 16.65		V = 4.16				R = 3.70
L, I, V	H = 2.43	A = 3.93	I = 3.47			M = 4.16	H = 3.47
	Y = 2.08	W = 2.08	A = 2.77	C = 2.50		L = 3.19	W = 2.77
			W = 2.08	R = 2.43	C = 2.77	Q = 2.08	L = 1.85
i	i + 1	i + 2	i + 3	i + 4	i + 5	i + 6	i + 7
	T = -2.88	P = -3.60	N = -3.60	D = -2.88	H = -1.92	D = -1.94	N = -2.88
	E = -4.81	D = -4.32	K = -4.32	E = -7.21	D = -2.88	G = -2.52	K = -4.32
	P = -12.97		P = -5.77		Q = -2.88		
			Y = -8.65				
DRB1*1101							
	W = 11	W = 9.77	M = 15.9	M = 4.83			
Y, F, W	R = 7.33	M = 3.06	L = 4.89	W = 3.66	R = 33	W = 8.56	R = 4.89
L, I, V	G = 3.26	Q = 2.75	V = 4.28	R = 2.14	K = 6.11	M = 4.89	W = 4.89
	H = 2.44	G = 1.96	W = 3.06	Q = 1.83	W = 2.44	R = 4.58	Q = 1.96
i	i + 1	i + 2	i + 3	i + 4	i + 5	i + 6	i + 7
	P = -2.29	D = -2.45		A = -2.45	N = -2.36	E = -3.27	
		I = -2.45		D = -3.27	A = -2.45	T = -4.91	
				E = -4.09	V = -2.45	D = -5.73	
					L = -3.27		
					S = -3.27		
					T = -5.73		
					E = -10.6		

^a See text for a detailed explanation on the calculation of the relative frequency of abundance for each amino acid at all positions. Basically, the frequency of abundance of an amino acid at a given position is the ratio between the frequency of abundance of the amino acid at this position in binder peptides (fbp), respect to the frequency in non binder peptides at the same position (fnbp). When the amino acid has an enhancing effect on binding, the ratio fbp/fnbp is taken as positive whereas if the amino acid has a deleterious effect on binding, the ratio fnbp/fbp is arbitrarily taken as negative. All binder peptides must have an anchor residue: Tyr, Phe, Trp, Leu, Ile, or Val at position **i**. Positive and negative values correspond to enhancing and deleterious amino acids respectively. A nil value is given to those amino acids having no effect on binding (not shown). The score is calculated by adding the individual contributions of amino acids at positions **i + 1** to **i + 7**.

The score of peptide YRELD AQH according to the DR1 motif would be:
Score = 7.6 = (3.06)+(-2.3)+(2.51)+(-2.36)+(2.39)+(0)+(4.30)

long to either of the above two groups, were considered as having no effect on binding.

RESULTS

In order to develop algorithms for the prediction of peptide binding to DR1, DRB1*0401 and DRB1*1101 molecules, we studied the ratios of relative abundance of amino acids in panels of well identified binder and non-binder peptides to these molecules. The procedure used is specified in Methods and the algorithms thus

obtained are shown in Table 1. These ratios of relative abundance were arbitrarily taken as positive or negative depending on whether the amino acid had an enhancing or deleterious effect on binding respectively. To calculate a score representing the binding tendency of a block containing an anchor amino acid (Y, F, W, L, I, V) at position **i**, the sum of the individual contributions of amino acids at positions **i + 1** to **i + 7** (the ratios between relative frequencies shown in Table 1) was taken. A nil value was given to those amino acids having no significant effect on binding. In this way the scores of all

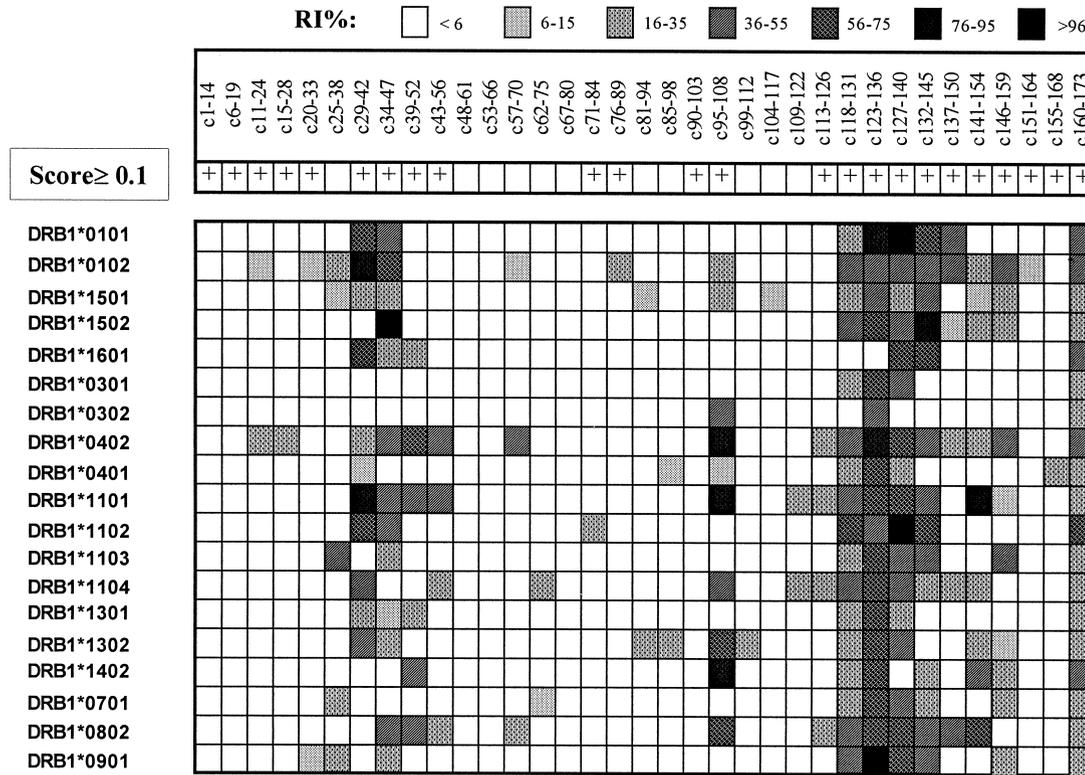


FIGURE 1 Relative binding capacity of 35 peptides from HCV core protein to 19 different HLA-DR molecules as studied by flow cytometry. This was calculated by the inhibition of the binding of biotinylated peptide HA(306-320) to HLA-DR molecules in competition experiments. The relative inhibitory capacity (RI%) of peptides was calculated according to the Formula:

$$RI\% = 100 \times (\text{signal inhibition with peptide tested}) / (\text{signal inhibition with unbiotinylated HA(306-320)})$$

The fluorescence intensities of triplicate samples were usually within 5% of the mean and always within 10%. See Methods for a detailed explanation of the calculation of RI%. A plus sign indicates that at a score ≥ 0.1 , the peptide is predicted as binder by the DRGen algorithm.

potential blocks contained by the peptides screened were calculated (see legend to Table 1 for a calculation example).

As shown below, in order to validate the three algorithms from Table 1, we tested their sensitivity and specificity against different panels of well identified binder and non-binder peptides reported by several research groups. We have defined the sensitivity and specificity of the predictions as the percentage of correctly predicted binder and non-binder peptides, respectively. We also tested these algorithms against 35, 15-mer synthetic peptides from HCV core protein whose binding ability to 19 HLA-DR molecules was measured by

flow cytometry (Fig. 1). Moreover, using the same panels of peptides, the sensitivities and specificities of these algorithms were compared with those attained with other algorithms reported in the literature [32, 40–42].

In Table 2, we compare the predictions made with the DR1 algorithm, deduced in the present publication, with another algorithm reported by Southwood et al. [42], which we adapted to predict binding using 8 amino acid blocks instead of 9 amino acid blocks reported by these authors. Calculations were carried out using the amino acid parameter values indicated in Fig. 2 of their publication but using only the first 8 amino acids. In Table 2, we compare the sensitivities and specificities of our algorithm with those attained with the modified algorithm of Southwood et al. [42]. For a given specificity, the sensitivity of our algorithm was higher than that attained with the modified algorithm of Southwood et al. [42], both against the set of 94 binder peptides to DR1, as well as to another set of 30 peptides that bind to DRB1*0101/0102 (taken from Prof. Ramensee data base published at web page: <http://www.uni-tuebingen.de/uni/kxi>) (Table 2). However, both algorithms behave similarly when tested against binder and non-binder peptides to DRB1*0101 from HCV core protein (Table 2).

In Table 3 we compare the predictions made with the algorithm DRB1*0401 deduced in the present publica-

TABLE 2 Sensitivity (%) and specificity (%) of two algorithms to predict binding of peptides to DR1. Effect of score on the sensitivity and specificity of the predictions

Algorithm	Score	Sensitivity against 94 BP ^a to DR1	Sensitivity against 30 BP ^b to DRB1*0101	Specificity against 71 NBP ^c to DR1	Sensitivity against 8 BP ^d to DRB1*0101	Specificity against 27 NBP ^e to DRB1*0101
DR1 (this work)	≥0.1	77.7	80.0	71.8	75	74.1
	≥2.0	75.5	73.3	81.7	75	74.1
	≥5.0	53.2	43.3	90.1	25	92.6
DRB1*0101 ^f (Southwood et al.)	≥0.01	68.1	73.3	32.4	100	59.2
	≥0.183 ^g	63.8	73.3	56.3	100	70.4
	≥1.57 ^h	52.1	66.7	71.8	62.5	85.5

BP = binder peptides, NBP = non-binder peptides.

^a 60 peptides from [29] plus 34 peptides from [33].

^b 30 binder peptides from Prof. Rammensee web page: <http://www.uni-tuebingen.de/uni/kxi>.

^c 71 non-binder peptides from [33] that had at least one core of 8 amino acids binding with an anchor residue at their N-terminus and that were used in the present work to deduce the algorithm to predict binding to DR1.

^d 8 binder peptides to DRB1*0101 from HCV core protein (this work, see Fig. 1).

^e 27 non-binder peptides to DRB1*0101 from HCV core protein (this work, see Fig. 1).

^f Adapted from [42] to predict binding using peptide blocks of 8 amino acids.

^g According to [42] threshold for prediction of 90% of the binders in their peptide library.

^h According to [42] threshold for prediction of 75% of the binders in their peptide library.

tion, with another four algorithms reported by others [32, 40–42], three of which were adapted to predict binding using the parameter values indicated by these authors, but the length was restricted to 8 amino acid blocks. Three panels of binder and non-binder peptides were used to compare these algorithms. The first panel contains some of the peptides used to deduce the DRB1*0401 algorithm of the present publication, the second panel corresponds to 27 binder and 41 non-binder peptides reported by Honeyman et al. [41], and the third panel corresponds to peptides from HCV core protein tested in the present publication (see data from Fig. 1). With respect to the data of Honeyman et al. [41], we considered the peptides as binder when they behave as such in their binding assay or in their biological assay, the remaining were considered as non-binders. With the exception of the algorithm of Honeyman et al. [41], which had a higher sensitivity and specificity for the prediction of their peptides, the algorithm DRB1*0401 developed in the present publication compares favorably with the other algorithms from Table 3 when tested against panel 1 and panel 2 of peptides. However, when these algorithms were tested against panel 3 (peptides from HCV core protein, the modified algorithm from Southwood et al. [42] was found to be more specific for a given sensitivity (Table 3).

Because, as shown in Fig. 1, some peptides may bind to different HLA-DR molecules, we decided to investigate the cross predictivity of each algorithm by testing it against peptides of their own restriction as well as against

peptides of the other two restrictions. As shown in Table 4, all three algorithms predicted peptide binding to their corresponding HLA-DR molecule with higher sensitivity. However, an important crossprediction for the other restrictions was observed. These results suggested to us to develop an algorithm for the degenerate prediction to several HLA-DR restrictions. As for the algorithms reported here, this general algorithm was deduced by comparing blocks of binder peptides with blocks of non-binder peptides. Thus, we compared blocks of binder peptides to DR1 (60 peptides [29]), DRB1*1101 (52 peptides [30]), DRB1*0401 (52 peptides [30]), DR1, DR2, DR5, DR52a (63 peptides [33]), and 47 peptides binding to a variety of HLA-DR molecules [9–12, 14, 31, 33, 43–55] with 43 peptides that were unable to bind to DR1, DR2, DR5 or DR52a [33]. This afforded the algorithm DRGen shown in Table 5.

To validate the DRGen algorithm we tested it against several panels of well characterized binder and non-binder peptides as well as against the 35, 15-mer peptides from hepatitis C virus core protein genotype 1b from Fig. 1. As shown in Fig. 1 and Table 6, the DRGen motif predicts the binding of core peptides to the panel of 19 HLA-DR molecules with a sensitivity of 75.9% and a specificity of 69.8% when the score was ≥0.1. If a score ≥2.0 was selected the sensitivity decreased to 62.1% but the specificity increased to 76.7%. We also validated our algorithm against several panels of peptides that have been reported to bind to a wide range of HLA-DR molecules (see Table 6). As shown in Table 6,

TABLE 3 Comparison of different algorithms for the prediction of binding to DRB1*0401 class II molecules on three panels of binder and non binder peptides. Effect of score on the percentage of sensitivity and percentage of specificity of the predictions

Algorithm	Score or IC50	Panel 1		Panel 2		Panel 3	
		BP ^a Sensi	NBP ^b Speci	BP ^c Sensi	NBP ^d Speci	BP ^e Sensi	NBP ^f Speci
DRB1*0401 (this paper)	Score \geq 0.1	90.0	67.4	100	48.8	75.0	37.0
	Score \geq 2.0	88.7	76.7	96.3	53.6	50.0	51.8
	Score \geq 5.0	73.7	86.0	70.4	75.6	12.5	70.4
Honeyman et al. ^g	Score \geq 6.0	NT	NT	74.1	85.4	NT	Nt
DRB1*0401 Adapted from Hammer et al. ^h	Score \geq 0.0	90.0	51.2	92.6	46.3	75.0	44.4
	Score \geq 2.0	80.0	83.7	55.5	90.2	12.5	88.9
	Score \geq 5.0	21.2	97.7	7.4	100	0	100
DRB1*0401 (Southwood et al. ⁱ)	Score \geq 0.250	76.2	48.8	74.1	53.7	75.0	85.2
	Score \geq 0.734 ^j	62.5	69.8	66.7	68.3	50.0	88.9
	Score \geq 2.617 ^k	41.2	83.7	44.4	92.7	25.0	96.3
DRB1*0401 Adapted from Marshall et al. ^l	IC50 \leq 5000	93.7	13.9	100	14.6	75.0	48.1
	IC50 \leq 73.5	76.2	69.8	66.7	70.7	37.5	88.9
	IC50 \leq 14.7	52.5	81.4	44.4	100	25.0	96.3

BP = binder peptides; NBP = non-binder peptides; Sensi = sensitivity; Speci = specificity.

^a 52 binder peptides from [29] plus 28 binder peptides from [32].

^b 43 non-binder peptides from [33] that do not bind to DR1, DR2, DR5, or DR52a.

^c 27 binder peptides from [41].

^d 41 non-binder peptides from [41].

^e 8 binder peptides to DRB1*0401 from HCV core protein (this paper, see Fig. 1).

^f 27 non binder peptides to DRB1*0401 from HCV core protein (this paper, see Fig. 1).

^g Algorithm of Honeyman et al. [41].

^h Algorithm adapted from [40] to predict binding using blocks of 8 amino acids.

ⁱ Algorithm adapted from [42] to predict binding using blocks of 8 amino acids.

^j According to [42] threshold for prediction of 90% of the binders in their peptide library.

^k According to [42] threshold for prediction of 75% of the binders in their peptide library.

^l Algorithm adapted from [32] to predict binding using blocks of 8 amino acids.

the sensitivity of the predictions against these panels is similar to that attained against panel 1 used to develop the algorithm. However, the specificity against panels 6 and 7 of non-binder peptides was lower than that attained against the non-binder peptides from panel 8 that was used to develop the algorithm.

DISCUSSION

By comparing blocks of peptides of 8 amino acids found in binder and non-binder peptides to different HLA-DR molecules, several algorithms were deduced to predict peptide binding to these molecules. As shown in Tables 2 and 3, when these algorithms were tested against several panels of peptides, it was found that they were in most cases able to predict binding of peptides to HLA-DR molecules with similar or higher sensitivity and specificity than other reported algorithms [32, 40,

42]. This was found, not only when the comparison was made against panels of peptides used to develop our algorithms, but most important, when independent panels of peptides were used. We believe that a key step in the deduction of our algorithms is the use of binder and non-binder peptides as well as correcting for the relative abundance of amino acids in proteins.

Since our algorithms are based on blocks of 8 amino acids, but the algorithm of Southwood et al. [42], of Hammer et al. [40], and of Marshall et al. [32] use 9, 9, and 11 amino acid blocks, respectively, we adapted their algorithms to the prediction using 8 amino acid blocks. This was done because when their algorithms were tested using blocks of 9 or 11 amino acids like in their original algorithm, the sensitivity of the predictions against the 9-mer peptides eluted from DR1 [29], DRB1*1101, and DRB1*0401 [30], was lower than the one attained using blocks of 8 amino acids (data not shown). Indeed, none of

TABLE 4 Sensitivity and specificity of different algorithms to predict binding of peptides to their corresponding MHC restriction as well as to other MHC restrictions. Effect of score on the sensitivity and specificity of the prediction using 8 amino acid blocks with an anchor residue at their N-terminus.

Algorithm	Score or IC50	Predicted sensitivity and specificity of binding (%)						
		Sensi against 94 BP ^a to DR1	Sensi against 52 BP ^b to DRB1*1101	Sensi against 80 BP ^c to DRB1*0401	Overall Sensi against 226 BPP ^d	Speci against NBP ^e	Sensi against BP ^f (this work)	Speci against NBP ^f (this work)
DR1	Score ≥ 0.1	77.7	59.6	67.5	69.9	71.8	52.9	72.2
	Score ≥ 2.0	75.5	51.9	55.0	62.8	81.7	47.1	72.2
	Score ≥ 5.0	53.2	36.5	21.2	38.0	90.1	17.6	94.4
DRB1*1101	Score ≥ 10	51.1	78.8	18.7	46.0	82.5	50.0	80.9
	Score ≥ 15	34.0	75.0	12.5	35.8	90.0	35.7	80.9
	Score ≥ 40	3.2	44.2	0	11.5	97.5	14.3	90.5
DRB1*0401	Score ≥ 0.1	77.6	71.1	90.0	80.5	67.4	75.0	37.0
	Score ≥ 2.0	68.1	69.2	88.7	75.7	76.7	50.0	51.8
	Score ≥ 5.0	48.9	38.5	73.7	55.3	86.0	12.5	70.4

BP = binder peptides, NBP = non-binder peptides, Sensi = sensitivity, Speci = specificity.

^a94 BP to DR1 (60 peptides from [29] plus 34 peptides from [33]).

^b52 BP to DRB1*1101 from [30].

^c80 BP to DRB1*0401 (52 peptides from [30] plus 28 peptides from [32]).

^d226 peptides from DRB1*0401, DRB1*1101 and DR1^{a, b, c}, respectively.

^eThese specificities of binding were calculated against the following sets of NBP:

for DR1: 71 NBP to DR1 reported by [33] and having an anchor at their N-terminus.

for DRB1*1101: 80 NBP to DR5 reported by [33] and having an anchor at their N-terminus.

for DRB1*0401: 43 NBP to DR1, DR2, DR5 or DR52a reported by [33] and having an anchor at their N-terminus.

^fThese sensitivities and specificities of binding were calculated using BP and NBP from Fig. 1:

for DR1 (17 peptides binding to DRB1*0101 or to DRB1*0102 or to both, as well as 18 peptides that did not bind to any of these two restrictions).

for DRB1*0101 (14 BP and 27 NBP from HCV core protein, see Fig. 1).

for DRB1*0401 (8 BP and 27 NBP from HCV core protein, see Fig. 1).

the 9 amino acid peptides reported by Hammer et al. would have been predicted by the original algorithm of Marshall et al. [32] that requires 11 amino acids for the predictions. Moreover, for the panels of peptides tested, the adapted algorithms shown in the present publication have a higher sensitivity of prediction than the unmod-

ified algorithms. Thus, we believe that these adaptations are not detrimental respect to the original algorithms.

As shown in Tables 2 through 4 the sensitivities and specificities of the different algorithms were dependent on the threshold scores used in the predictions. At high scores the algorithms became more specific (higher per-

TABLE 5 DRGen binding motif. Ratios of relative frequency of abundance of amino acids of binding relevance^a

		A = 2.78					H = 3.45
Y,F,W	R = 8.76	W = 2.15	M = 7.61				V = 3.09
L,I,V	W = 2.23	R = 2.06	L = 3.06	R = 1.94	R = 9.19		R = 2.83
	Y = 1.72	K = 2.01	A = 1.80	M = 1.87	K = 2.94	R = 1.83	W = 1.72
i	i + 1	i + 2	i + 3	i + 4	i + 5	i + 6	i + 7
	C = -5.22	P = -1.93	D = -2.05	P = -1.74	E = -4.31	S = -1.80	S = -2.51
	P = -7.83	D = -2.98	T = -2.32	E = -1.83	H = -4.35	D = -4.06	F = -2.78
		E = -3.31	E = -2.61	D = -1.86			D = -3.80
			C = -2.78				

^aSee text for a detailed explanation on the calculation of the relative frequency of abundance for each amino acid at all positions. Basically, the frequency of abundance of an amino acid at a given position is the ratio between the frequency of abundance of the amino acid at this position in binder peptides (fbp), respect to the frequency in nonbinder peptides at the same position (fnbp). When the amino acid has an enhancing effect on binding, the ratio fbp/fnbp is taken as positive whereas if the amino acid has a deleterious effect on binding, the ratio fbp/fnbp is arbitrarily taken as negative. All binder peptides must have an anchor residue: Tyr, Phe, Trp, Leu, Ile, or Val at position **i**. Positive and negative values correspond to enhancing and deleterious amino acids, respectively. A nil value is given to those amino acids having no effect on binding (not shown). The score is calculated by adding the individual contributions of amino acids at positions **i+1** to **i+7**. The score of peptide FRGDRKSH would be: Score = 13.24 = (8.76)+(0)-(2.05)+(1.94)+(2.94)-(1.80)+(3.45) for a block of 8 amino acids.

TABLE 6 Validation of the algorithm DRGen against different panels of binder and non-binder peptides. Effect of score on the sensitivity and specificity of the predictions

Score	Sensitivity (%)					Specificity (%)		
	1 ^a	2 ^b	3 ^c	4 ^d	5 ^e	6 ^f	7 ^g	8 ^h
Score \geq 0.1	84.7	84.4	81.5	87.3	75.9	51.2	66.7	69.8
Score \geq 2.0	81.4	76.7	66.7	78.8	62.1	58.5	66.7	76.7

Panel 1 and panel 8, in bold, and panel 2 to panel 7 were used to develop and validate the algorithm, respectively.

^aPanel of 232 BP from [33] and others (see text from results section).

^bPanel of 90 BP from [31] eluted from different HLA-DR molecules (1, 11, 16, 11, 18, and 33 peptides eluted from DR1, DR2, DR3, DR4, DR7, and DR8, respectively).

^cPanel of 27 BP from [41].

^dPanel of 165 BP from Prof. Rammensee web page: <http://www.uni-tuebingen.de/uni/kxi> (37, 13, 12, 18, 25, 7, 8, 4, 8, 15, 15, 3 peptides from: HLA-DRB1*0301, HLA-DRB1*0901, HLA-DRB1*1201, HLA-DRB1*1301, HLA-DRB1*1302, HLA-DRB1*1401, HLA-DRB1*1501, HLA-DRB1*1502, HLA-DR3, HLA-DRB5*0101, HLA-DR2 (DRB5*0101 or DRB1*1501, and HLA-DR7) respectively).

^ePanel of 29 BP from HCV core protein from the present study (see Fig. 1).

^fPanel of 41 NBP from [41].

^gPanel of 6 NBP from HCV core protein from the present study (see Fig. 1).

^hPanel of 43 NBP from [33].

centage of correctly predicted non-binder peptides) but at the expense of an important drop in sensitivity (lower percentage of correctly predicted binder peptides). Thus, the choice of the threshold level will depend on the viability of experimentally testing the binding of the peptides to MHC molecules or their TD_h character.

As shown in Fig. 1 as well as in data reported in the literature [12, 56] some peptides are able to bind to different class II molecules, showing degeneracy in binding. For this reason, we decided to study the level of crosspredictivity of our algorithms. As shown in Table 4 when the algorithms for DR1, DRB1*1101, and DRB1*0401 were tested, it was found that although each algorithm was more sensitive against peptides of its own restriction, a great deal of cross prediction was observed. The algorithm with less cross prediction was the one for DRB1*1101, which at scores \geq 40, when tested against peptides known to bind to DRB1*1101 and against two other panels known to bind to DR1 or to DRB1*0401, predicted binding with sensitivities of 44.2%, 3.2%, and 0%, respectively. This low crossprediction may be related to the superior specificity of this algorithm. Indeed, for scores \geq 40, 78 of the 80 peptides that did not bind to DR5 [33] were predicted as non-binder, which corresponds to a specificity of 97.5%. (DR5 is an alternative nomenclature for DR11.) This type of crossrecognition has been reported for other HLA-DR molecules. Thus, based on the sequence of

eluted peptides from DR2, DR3, DR4, DR7, and DR8, Chicz et al. [31] proposed DR-specific peptide motifs. However, the motif described for a given DR restriction can also be found in peptides eluted from other DR molecules (Table 8 from Chicz et al. [31]). Thus, the sequence alignment for DR2 suggested by Chicz et al. is also fulfilled by 23.6% of peptides eluted from the remaining four other DR molecules. Similarly, the sequence alignments for DR3, DR4, DR7, and DR8 are fulfilled by 57.2%, 27.3%, 59.7%, and 32.7% of peptides eluted from the remaining DR molecules respectively (data not shown). This shows that eluted peptides may contain more than one DR specificity.

The observation that the same peptide could be predicted by algorithms for different HLA-DR restrictions, as well as the results from Chicz et al. [31] discussed above, prompted us to develop a motif for the degenerate prediction of binding to several HLA-DR restrictions. It was also thought that this motif might be a useful tool in vaccine development for the selection of those regions of an antigen with the capacity of binding to several HLA-DR restrictions. Thus, as described earlier, we deduced the algorithm DRGen shown in Table 5. This algorithm was then validated using several panels of peptides characterized by others [31, 33, 41], (Prof. Rammensee), as well as against 35 peptides from HCV core protein tested in the present study (Fig. 1). This validation showed similar sensitivities and specificities than those attained using the panels of peptides used to develop the DRGen algorithm (Table 6). These results suggest that the DRGen algorithm may be a useful tool to predict binding to HLA-DR molecules.

As shown in Fig. 1, 22/29 (75.9%) of binder peptides from hepatitis C virus core protein genotype 1b were predicted as such by the DRGen algorithm. It is interesting to note that none of the peptides, which were able to bind to several HLA-DR molecules, was left unpredicted. Indeed, peptides that were not predicted were either non-binder or peptides that bound to few of the 19 HLA-DR molecules tested. These results show that those peptides that are able to bind to many HLA-DR molecules are efficiently predicted by the DRGen motif. Of the 6 non-binder peptides 4 were predicted as non-binder but the other 3 were predicted as binder.

Other researchers [25, 26] have published algorithms for the prediction of binding to MHC class II molecules. When we tested their algorithms using the program "T sites" [57] it was found that for a given specificity the sensitivity of these algorithms was well below that attained by the DRGen algorithm (data not shown).

In Table 7 we compare the role of amino acids on binding of peptides to HLA-DR molecules reported by others [33, 34, 44, 54, 58–61] with that of the general DRGen binding motif. This shows that most of the

TABLE 7 Effect of amino acids at positions **i** to **i+7** on binding of peptides to MHC class II molecules

i	i+1	i+2	i+3	i+4	i+5	i+6	i+7	Ref
Y ↑ F ↑ W ↑ Y ↑ F ↑ W ↑					K ↑ R ↑		K ↑ R ↑	[54] [34]
	Absence of negative interactions at all positions, more important than presence of positive interactions							[58]
	Enhanced binding of peptide HEL (104-120) at acidic pH							[59]
Y ↑		E ↓ D ↓	D ↓	D ↓ E ↓	D ↓	E ↓ D ↓	D ↓	[44] [60] [12] [61]
Y,F,W,I,L,V anchor	R ↑ W ↑ Y ↑ C ↓ P ↓	A ↑ K ↑ R ↑ W ↑ D ↓ E ↓ P ↓	A ↑ L ↑ M ↑ C ↓ D ↓ E ↓ T ↓	M ↑ R ↑ D ↓ E ↓ P ↓	K ↑ R ↑ E ↓ H ↓	R ↑ D ↓ S ↓	H ↑ R ↑ V ↑ W ↑ D ↓ F ↓ S ↓	This work

↑ Enhancing effect on binding.

↓ Deleterious effect on binding.

effects reported are compatible with our peptide binding motif. It is clear that, in addition to an anchor residue, binder peptides should have few, if any, amino acids giving negative interactions with the MHC. It has been suggested that the absence of “negative” amino acids may be more important than the presence of amino acids giving a “positive” interaction with MHC [58]. Also, the reported enhanced binding of peptide HEL(104–120) at acidic pH [59], where the net charge is less negative, is in agreement with the disrupting effect on binding of Asp and Glu at several positions of the peptide at higher pH [60] where the net charge is more negative.

The crystal structure of an HLA-DR1 molecule complexed with peptide HA(306-318) shows that residues Tyr308, Gln311, Thr313, Leu314, and Leu316 of HA(306-318) interact respectively with pockets 1, 4, 6, 7, and 9 of the HLA-DR1 molecule (See Fig. 4 from publication of Stern et al. [62]). If we consider that Tyr308 corresponds to the anchor at position **i** of our peptide motif, then pockets 1, 4, 6, 7 and 9 of the MHC may interact with positions **i**, **i+3**, **i+5**, **i+6**, and **i+8** of the motif, respectively. Thus, the enhancing or disrupting effect of amino acids at positions **i+3**, **i+5**, **i+6**, and **i+8** of the motif should be compatible with the amino acids delimiting pockets 4, 6, 7, and 9 of the MHC, respectively. Thus, pocket 1 is formed mainly by hydrophobic residues [62]. This is compatible with the interaction with the reported hydrophobic anchor :Tyr, Phe, Trp, Leu, Ile, Val at position **i**.

Pocket 4 may interact with residue **i+3** of the DRGen motif which favours Met and Leu at this position. This preference may be related with packing of Met or Leu with the hydrophobic residues Phe/His/Tyrβ13 (of some DR molecules) and Tyrβ78 in the pocket. The deleterious effect of Asp and Glu may be related with an electrostatic repulsion against a negatively charged amino

acid close to pocket 4, like Gluβ28 in DR1, and Aspβ28 in the remaining DR molecules.

Positions **i+5**, **i+6**, and **i+8**, which interact with pockets 6, 7 and 9 of the MHC molecule, do not favor Asp residues (Table 5) suggesting the presence of negatively charged residues at/or near these MHC pockets. For instance Glu α11 and Asp α66 from pocket 6 and Asp β57 from pocket 9 and/or with Glu β9 in most DR molecules. The presence of these negatively charged residues of the MHC molecule, might also explain the enhancing effect on binding of Arg in those positions where the negatively charged residues have a deleterious effect. Thus, the general amino acid topology of the binder peptide DRGen motif favors hydrophobic and positively charged amino acids, which have chemical affinity for hydrophobic and negatively charged amino acids respectively (Table 8). Also, the strong deleterious effect due to Pro, at position **i+1** may be related to the reported effect on the orientation of the peptide chain by Pro [63, 64] that may prevent the amino acids from the peptide to interact with the HLA-DR molecule.

To summarize, the published HLA-DR structure [62] suggests that its general topology may be complementary to the topology associated with the proposed DRGen peptide binding motif. This motif is able to predict with high sensitivity and specificity those regions of an antigen that may bind to HLA-DR molecules. This prediction, in conjunction with a better understanding of antigen processing and of immunodominancy, due to higher affinity of peptides for HLA-DR molecules and/or higher affinity of the complex: peptide-(class II molecule) by the T cell receptor, may allow in not too distant future, the identification of the most relevant TD_h from antigens. This is of paramount importance because TD_h are essential for the induction of humoral and cellular

immune responses, and consequently, for the design of future vaccines.

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