Relationship between kinetic stability and immunogenicity of HLA-DR4/peptide complexes

Frances C. Hall[1], Joshua D. Rabinowitz[2], Robert Busch[3], Kevin C. Visconti[1], Michael Belmares[2], Namrata S. Patil[3], Andrew P. Cope[1], Salil Patel[1], Harden M. McConnell[2], Elizabeth D. Mellins[3] and Grete Sonderstrup[1]

[1] Department of Microbiology and Immunology, Stanford University, Stanford, USA
[2] Department of Chemistry, Stanford University, Stanford, USA
[3] Department of Pediatrics, Stanford University, Stanford, USA

Immunodominant T cell epitopes from the autoantigen human cartilage glycoprotein 39 have previously been mapped in the context of HLA-DR*0401 and *0402, using mice expressing HLA-DR4 transgenes. We measured the dissociation rates of these epitopes from soluble recombinant DR*0401 and DR*0402 to assess the relationship between peptide/HLA-DR4 kinetic stability and immunogenicity. Experiments were performed at endosomal pH (5.5) and at cell surface pH (7), in the absence and presence of soluble recombinant HLA-DM (sDM). All (4/4) immunodominant peptide/HLA-DR complexes exhibit dissociation half-times of 1 h to several days. In contrast, most (3/4) non-immunodominant complexes dissociate with half-times <30 min under at least one of these conditions. Interestingly, a complex which is stable except in the presence of HLA-DM at pH 5.5 is immunogenic only following peptide immunization, while a complex which is stable at acidic but not at neutral pH, is non-immunogenic following either whole protein or peptide immunization. These data indicate that kinetic stability of peptide/MHC complexes *in vivo* is a key determinant of immunogenicity.

Key words: T lymphocyte / MHC / Antigen/peptide/epitope / Antigen presentation

1 Introduction

The observation that small changes in the structure of MHC class II proteins can alter predisposition to autoimmunity suggests a role for antigen presentation in the pathogenesis of disease. For example, HLA-DR*0401 is associated with susceptibility to rheumatoid arthritis (RA), whereas the closely related MHC protein HLA-DR*0402 is not [1]. We have previously studied the immunogenicity of the synovial autoantigen human cartilage glycoprotein-39 (HCgp-39) in the context of DR*0401 and DR*0402 using mice expressing HLA-DR4 and human CD4 transgenes [2]. The specificities of the T cell hybridomas raised by immunizing the transgenic

The last three authors have contributed equally to this work.

Abbreviations: DR*0401: Heterodimeric product of HLA-DRA1*0101 and HLA-DRB1*0401 **DR*0402:** Heterodimeric product of HLA-DRA1*0101 and HLA-DRB1*0402 **sDR:** Soluble recombinant HLA-DR **sDM:** Soluble recombinant HLA-DM **CLIP:** Class II-associated invariant peptide **HCgp-39:** Human cartilage glycoprotein-39 **MBP:** Myelin basic protein

DR*0401 and *0402 mice with HCgp-39 revealed that three HCgp39 peptides, 262–276, 100–115 and 322–337, are immunodominant in the DR*0401 mice. Two different peptides, 298–313 and 22–37, are immunodominant in the DR*0402 mice (Table 1).

Previous studies of peptide binding to MHC class II molecules indicate that high-affinity peptide/MHC complexes tend to be immunogenic [3–6], although some exceptions to this rule have been documented, including myelin basic protein (MBP) Ac1–11 and MBP (111–129) [4, 7, 8]. The competitive binding assays used in the majority of these studies provide a relative measure (IC_{50} value), representing a composite of the association and dissociation rates of the peptide/MHC complex. Since the association rate constants of many peptides to active, empty MHC at a given pH are all approximately equal to one another ([9] and F.H., unpublished), stability with respect to dissociation correlates strongly with thermodynamic stability in the case of peptide-MHC binding [$\Delta G = -RT \ln k_{off}/k_{on}$, where R is the gas constant and T is temperature (K)]. It is, therefore, reasonable to consider only dissociation rate constants in comparing the immunogenicity of different peptides, and stability in this report refers to stability with respect to dissociation. The

sensitivity of relative peptide/MHC complex con-centrations to dissociation half-times depends on the environment. For example, if peptide-MHC reactions were to reach equilibrium in an endosomal compartment, the relative concentrations of complexes would simply be proportional to the ratio of the corresponding half-times for dissociation. In contrast, if the complexes were to translocate to a cell surface lacking free peptide in the environment, then a twofold increase in dissociation half-time could correspond to as much as a factor of 1,024 increase in cell surface complex concentration after ten of the longer half-times. Thus, the relative immunogenicity of different peptides can be expected to depend on their relative kinetic stability and, under some circumstances, might be highly sensitive to this "stability".

The immunogenicity of a T cell epitope is the outcome of processing/presentation in the antigen-presenting cell (APC) as well as the T cell response. An immunogenic peptide usually associates with an MHC class II mole-cule in an endosomal compartment, survives editing by HLA-DM, and remains sufficiently stable on the cell sur-face to be recognized by a specific TCR. Alternatively, antigen present in the form of extracellular peptide can load directly on the cell surface [10]. Peptide/MHC class II complexes on the cell surface may be subjected to fur-ther rounds of editing mediated by HLA-DM, at neutral pH, either on the cell surface itself [11, 12], or as com-plexes from the cell membrane are recycled via weakly acidic early endosomal compartments [13]. The duration of ligation of specific TCR by peptide/MHC ligand is an important determinant of the T cell response. Although acid flux can be detected in a T cell within minutes of recognition by its TCR of peptide/MHC ligand [14], previ-ous studies have suggested that TCR ligation is required for approximately 20 h to stimulate proliferation in naive T cells. In contrast, effector T cells are committed to pro-liferation within 1 h of TCR ligation [15]. The nature and the activation state of the APC influence not only the threshold for T cell commitment to proliferation but also T cell differentiation [16]. T cell activation is, therefore, influenced by many factors including the duration of liga-tion and the steady-state occupancy of the TCR. This will be determined by the local density of MHC mole-cules on the APC, the avidity of the TCR for the peptide/

Table 1. Immunogenicity of HCgp-39 protein sequences in HLA-DR4 transgenic mice[a]

Sequence from HCgp-39 protein	HLA-DR restriction	Percentage specific T cell hybridomas[b]	Immunogenicity[c]
(100–115)	*0401	20	**Major**
NFGSQR**FSKIASNTQS**	*0402	<1	Minor
(262–276)	*0401	34	**Major**
GRS**FTLASSETG**VGAP	*0402	<1	Minor
(322–337)	*0401	27	**Major**
GNQWVG**YDDQESVKS**K	*0402	0	Non-immunogenic
(22–37)[d]	*0401	0	Peptide-only
YKL**VCYYTSWSQ**Y	*0402	32	**Major**
(298–313)	*0401	0	Peptide-only
EICDF**LRGATVHRT**LG	*0402	54	**Major**

a) Residues occupying the P1–P9 pockets of the MHC proteins are shown in bold. Alignments of peptides 22–37, 100–115 and 262–276 are from [2]. Other alignments are from data described in Sect. 2.

b) Data reproduced by permission from Arthritis and Rheumatism; showing frequency of peptide-specific T cell hybridomas expressed as a percentage of HCgp-39-specific T cell hybridomas raised in DR*0401 transgenic mice (*n*=212), and specific hybridomas generated in DR*0402 mice (*n*=151), following immunization with whole HCgp-39 protein.

c) A major epitope is defined as one recognized by at least 20% of the T cell hybridomas specific for HCgp-39 following whole protein immunization. A minor epitope is recognized by an occasional T cell hybridoma raised by whole protein immunization. A peptide-only epitope is recognized by zero hybridomas following whole protein immunization, but produces T cell hybridomas specific only for peptide following peptide immunization. A non-immunogenic epitope is recognized by zero hybridomas following either whole protein or peptide immunization.

d) Due to low solubility in water, peptide 22–37 was not studied in dissociation assays.

MHC ligand and the kinetic stability of the peptide/MHC complex.

To examine the importance of kinetic stability, we measured peptide/MHC complex dissociation kinetics using purified, soluble, recombinant proteins and fluorescently labeled synthetic peptides. Dissociation kinetics measured in this way have previously been shown to correlate well with peptide/MHC complex stability on live cells [17]. These experiments provide additional information beyond that obtained from the previously reported IC_{50} values, in that they measure the dissociation rate in isolation from the association rate of a complex, thereby enabling a comparison of the stabilities of peptide/MHC complexes involving different MHC molecules. In addition, these data compare complex stabilities at pH 5.5 and pH 7, in the absence and presence of soluble recombinant HLA-DM (sDM), and indicate how the range of conditions encountered in the antigen presentation pathway may influence the cumulative kinetic stability of a complex.

2 Results

2.1 Peptide/MHC complex dissociation at pH 5.5

Previously, HLA-DR4 transgenic mice were immunized with HCgp-39 and T cell hybridomas were raised. The specificities of T cell hybridomas indicate that peptides 262–276, 100–115 and 322–337 are immunodominant in the context of DR*0401 (Table 1). Peptides 262–276 and 100–115 are also weakly immunogenic in the DR*0402 mice, whereas 322–337 is not immunogenic in the DR*0402 mice immunized with either free peptide or whole protein. Peptides 298–313 and 22–37 are immunodominant in the DR*0402 mice. Peptide 298–313 is immunogenic in the DR*0401 mice immunized with free peptide but not whole protein, with the hybridomas generated by free peptide immunization recognizing only free peptide and not whole protein ("peptide-only" T cells). We have attempted to form each of these peptide/DR4 complexes *in vitro*. The peptide 22–37 has very low water solubility and, therefore, was not studied further. The other peptide/DR4 complexes readily formed, and their dissociation half-times at pH 5.5 were measured.

Peptides f262–276, f100–115 and f322–337 are all more stable when associated with DR*0401 than with DR*0402 at pH 5.5 (Fig. 1), in keeping with the immunodominance of these epitopes in the context of DR*0401. In contrast, peptide f298–313 is more stable on DR*0402 than DR*0401 at pH 5.5, consistent with the immunodominance of this epitope in the context of DR*0402 (Fig. 1).

The immunogenicity data summarized in Table 1 indicate that, of the three peptides that are immunodominant in the mice expressing DR*0401, two are also minor epitopes in the context of DR*0402 (262–276 and 100–115). In contrast, peptide 322–337 is not immunogenic in the mice expressing DR*0402, irrespective of whether the mice were immunized with whole HCgp-39 or free 322–337 peptide. The dissociation experiments performed at pH 5.5 do not provide any insight into the mechanism for this discrepancy in immunogenicity; indeed the f322–337/*0402 complex is more stable at pH 5.5 than the f100–115/*0402 complex.

2.2 Peptide/MHC complex dissociation at pH 7

At pH 7, the three peptides immunodominant in the mice expressing DR*0401 are again more stable in association with soluble DR*0401 than with DR*0402 (Table 2). Similarly, the 298–313 peptide, which is immunodominant in the mice expressing DR*0402, is more stable in association with soluble DR*0402 than with DR*0401. Consistent with prior reports, the dissociation rate at pH 7 is comparable to or slower than at pH 5.5 for most of the studied peptide/MHC complexes. Surprisingly, however, peptide 322–337 dissociates much more rapidly at pH 7 than at pH 5.5 from either DR*0401 or DR*0402 (acceleration of 40- and 70-fold, respectively). This results in peptide 322–337 dissociating from DR*0401 with a half-time of 10 h at pH 7, and from DR*0402 with a half-time of only 8 min at pH 7. Thus, while both complexes are similarly destabilized at pH 7 relative to pH 5.5, the absolute stabilities of the two complexes are consistent with the 322–337 epitope being immunodominant in the DR*0401 mice, but not being immunogenic in the DR*0402 mice. In particular, the rapid dissociation of the 322–337 peptide from DR*0402 at pH 7 suggests an explanation for the failure of this epitope to trigger an immune response in the DR*0402 mice, even following free peptide immunization.

2.3 Peptide/MHC complex dissociation at pH 5.5 in the presence of HLA-DM

Relative dissociation rates at pH 5.5 in the presence of HLA-DM follow a similar pattern to those at pH 5.5 in the absence of DM (Fig. 1; Table 2). Dissociation of each peptide/MHC complex is accelerated by 0.4 µM sDM, with the degree of acceleration ranging from less than 1.5-fold for peptide 262–276 bound to DR*0401 to greater than 100-fold for peptide 298–313 bound to DR*0402. Again, there is a strong trend for the immunogenic complexes to be more stable.
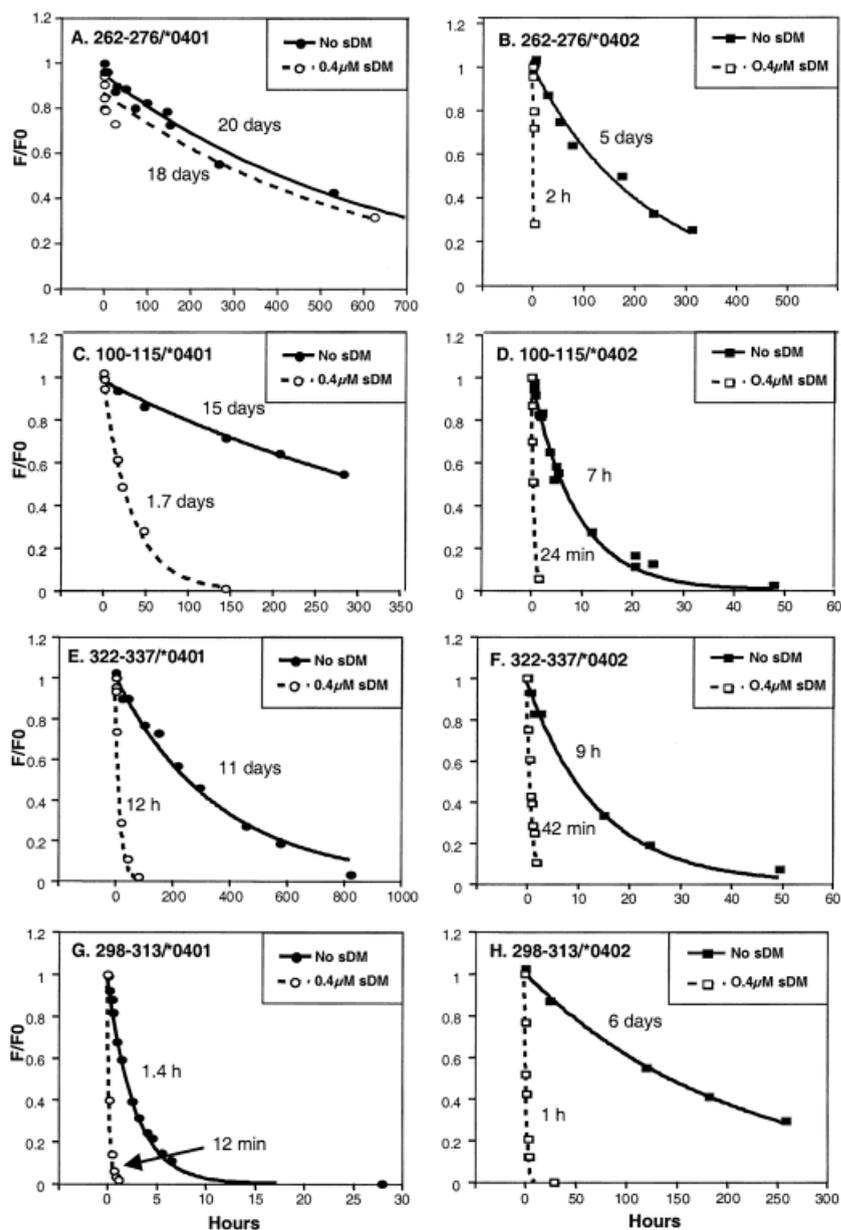
Fig. 1. Dissociation of HCgp-39 peptides from DR*0401 and DR*0402 at pH 5.5. Peptide/DR4 complexes were allowed to disso-ciate at pH 5.5 and 37°C in the presence of unlabeled competitor peptide and in the presence (open symbols) or absence (closed symbols) of 0.4 μM sDM. Plotted data indicate the proportion of the initial f-peptide/DR4 complex (F/F0) remaining after the given dissociation time. Lines indicate best fits to a single exponential decay function with the indicated dissociation half-time.

Beyond confirming the patterns seen at pH 5.5 in the absence of DM, the dissociation measurements in the presence of HLA-DM provide additional insight into the immunogenicity pattern of the peptide 298–313 in the DR*0401 mice. This peptide is non-immunogenic follow-ing whole protein immunization of these mice, but does stimulate T cells after immunization of DR*0401 mice with free peptide. The 298–313 peptide dissociates from DR*0401 reasonably slowly at pH 7 (half-time of 4 h),

consistent with the ability of free peptide immunization to trigger a T cell response. In contrast, it dissociates very rapidly at pH 5.5 in the presence of 0.4 μM sDM (half-time of 12 min), consistent with HLA-DM editing these complexes from endosomes following whole protein immunization. Thus, instability of a peptide/MHC com-plex in presence but not absence of DM is one cause of "peptide-only" T cells.

Table 2. Dissociation half-times of HCgp-39 peptide/DR4 complexes in hours[a]

	Peptide	pH 5.5		pH 7	
		– sDM	+ sDM	– sDM	+ sDM
DR*0401	f100–115	355 ± 0	41 ± 16	235 ± 24	10 ± 0.3
	f262–276	490 ± 40	425 ± 13	1,779 ± 159	1,526 ± 30
	f322–337	255 ± 5	12 ± 0.3	10 ± 0.4	12 ± 0.4
	f298–313	1.4 ± 0.2	*0.2 ± 0*	4.4 ± 0.2	5 ± 0.9
DR*0402	f100–115	6.8 ± 0.4	*0.4 ± 0*	5.8 ± 0.4	1.9 ± 0.1
	f262–276	124 ± 14	1.7 ± 0.07	27 ± 6	13 – 1.5
	f322–337	9 ± 0.8	0.7 ± 0.05	*0.13 ± 0*	0.17 ± 0
	f298–313	135 ± 17	1.3 ± 0.1	141 ± 73	54 ± 4

a) Peptide/DR4 complexes were formed at pH 5.5 and allowed to dissociate at either pH 7 (cell surface pH) or pH 5.5 (endosomal pH), in the absence or presence of 0.4 μM sDM. The measurements represent the mean and standard error of the dissociation half-time computed from two or more independent dissociation curves obtained on different days. The conditions where instability of a complex corresponds to poor immunogenicity are indicated in italics.

2.4 Peptide/MHC dissociation at pH 7 in the presence of HLA-DM

The presence of 0.4 μM sDM at pH 7 markedly accelerates the dissociation of some peptide/MHC complexes, while having no apparent effect on others. The 262–276/DR*0401 complex is virtually resistant to the effect of sDM at pH 7. Its remarkable stability under all conditions tested may account for its apparently high immunogenicity *in vivo*, being recognized by 34% specific, DR*0401-restricted T cell hybridomas. Although the 322–337/*0401 complex is 25-fold less stable at pH 7 than at pH 5.5, its dissociation at pH 7 is not further accelerated by the presence of sDM. In contrast, the 100–115/*0401 complex is highly DM-sensitive at pH 7 and accelerates over 20-fold more rapidly. These differential sensitivities to sDM at pH 7 may account for the hierarchy of immunogenicity, *in vivo*, among the three immunodominant peptide/DR*0401 complexes, namely 262–276/DR*0401 > 322–337/*DR*0401 > 100–115/DR*0401. Interestingly, the 298–313/*0401, complex is DM resistant at pH 7. The free peptide 298–313 is immunogenic in the DR*0401 transgenic mice; presumably it can load on the cell surface where it would be relatively resistant to DM editing.

The peptide complexes with DR*0402 exhibit similar variation in DM-sensitivity at pH 7. Notably, the 322–337/*0402 complex, which is intrinsically unstable at pH 7, is not further destabilized by the presence of sDM. The observation that 322–337/DR*0401 is also DM resistant at pH 7 suggests that characteristics of this

peptide, which underlie its pH sensitivity, may also confer DM resistance at neutral pH. The other peptide/DR*0402 complexes are all DM sensitive at pH 7, exhibiting a range of between 2- and 3-fold acceleration of dissociation. The immunodominant complex, 298–313/DR*0402, is approximately 4- and 26-fold more stable, at pH 7 in the presence of sDM, than the weakly immunogenic complexes, 262–276/DR*0402 and 100–115/DR*0402, respectively.

2.5 Structural basis for the dissociation kinetics

The peptide core motif required for MHC binding is only 9 amino acids long, each of the 16-mer peptides studied could therefore bind in several different registers. However, the dissociation kinetics of each of the studied complexes is monophasic, consistent with detectable binding occurring in only a single register. Peptides 100–115 and 262–276 bind to both DR*0401 and *0402 with a particular peptide phenylalanine occupying the P1 MHC pocket, as previously shown (see Table 1). Peptide 322–337 dissociates from both DR*0401 and DR*0402 with similar kinetics to the truncated peptide 327–337, demonstrating that binding to both MHC alleles occurs with peptide tyrosine 328 in the P1 MHC pocket. Peptide 298–313 has been previously proposed to bind to both DR*0401 and DR*0402 with phenylalanine 302 in the P1 MHC pocket. However, we find that mutation of this phenylalanine to alanine only slightly alters the dissociation kinetics of this peptide from either MHC allele; f-302A/0401 dissociates with a half-time of (mean ± SE)

2.5±0.09 h at pH 5.5 and f-302A/*0402 dissociates with a half-time of 206±10 h. In contrast, mutation of residue 303 leucine to alanine results in poor association of f-303A to either DR*0401 or DR*0402. The poor initial signal obtained precludes quantitation of the dissociation rate of these complexes; however, for f298–313(303A)/*0402 the small amount of complex formed appears to dissociate with a half-time of 1 h (compared with 135 h for the wild-type peptide). Thus peptide 298–313 binds to both *0401 and *0402 with leucine 303 in the P1 MHC pocket.

Based on these register assignments, the three peptides that are immunodominant on DR*0401 each have an aromatic residue occupying the P1 MHC pocket, whereas the peptide that is immunodominant on DR*0402 has an aliphatic residue, leucine, in the P1 pocket. This pattern is consistent with DR*0401 and DR*0402 differing at position beta-86 (glycine in *0401, valine in *0402), resulting in DR*0401 having a larger P1 pocket [18]. In contrast, no particular pattern is noted at the P4 pocket, where the differences between *0401 and *0402 that are believed to be responsible for rheumatoid arthritis (RA) susceptibility localize [1].

3 Discussion

Immunogenicity is the outcome of a series of processes. Antigens are taken up into endosomal compartments of specialized cells (APC), where unfolding and proteolysis of the antigen coincides with the availability of an active state of the empty MHC class II protein after release of CLIP peptide [19, 20]. Antigenic peptides bind to empty MHC. An enzyme present in endosomes, HLA-DM, both stabilizes the active state of empty MHC and catalyzes dissociation of antigenic peptides [21]. Once formed, peptide/MHC complexes that survive editing by HLA-DM move to the cell surface, where they are examined by TCR. We hypothesized that the kinetic stability of peptide/MHC complexes, through serial stages of antigen processing, was a key determinant of immunogenicity. We therefore studied eight peptide/DR4 complexes for which patterns of immunogenicity had previously been defined *in vivo*.

Consistent with peptide/MHC dissociation kinetics being a key determinant of immunogenicity, each of the three peptides known to be immunodominant in the context of DR*0401 was more stable complexed to DR*0401 than to DR*0402, under all conditions studied. Conversely, the peptide 298–313, which is immunodominant in the context of DR*0402 was more stable in complex with DR*0402 than DR*0401. The immunodominant complex 262–276/DR*0401 proved extremely stable with disso-

ciation half-times greater than 400 h, under all conditions studied. Two other immunodominant complexes, 100–115/DR*0401 and 322–337/DR*0401 exhibited intermediate stability, with dissociation half-times ranging between 10 and >200 h, depending on the condition studied. In contrast, three complexes were clearly unstable, with a dissociation half-time of less than 30 min under at least one of the four conditions studied. Of these, complex 100–115/DR*0402 is weakly immunogenic, complex 322–337/DR*0402 is non-immunogenic and complex 298–313/DR*0401 is immunogenic in DR*0401 mice only following immunization with free peptide. Importantly, the 298–313/DR*0401 complex exhibits a longer dissociation half-time of >4 h at pH 7, either in the absence or presence of sDM. In each of these six complexes, the kinetic stability is consistent with the pattern of immunogenicity *in vivo*.

The immunogenicity of the two remaining complexes cannot be explained by kinetic stability alone. A weakly immunogenic complex, 262–276/DR*0402 exhibited dissociation half-times between 1.7 and 124 h, being least stable at pH 5.5, in the presence of sDM. Surprisingly, the immunodominant complex, 298–313/DR*0402 exhibited even lesser stability at pH 5.5 in the presence of sDM, with a dissociation half-time of only 1.3 h under this condition. It did, however, prove more stable at pH 7 than 262–276/DR*0402. If DR*0402 loading occurred preferentially in early endosomal compartments, at neutral pH, the immunodominance of 298–313 could be explained; this is a testable hypothesis. Interestingly, the expression of HLA-DM in B cells from patients with RA has been reported to be reduced, compared with healthy subjects or inflammatory controls [22]. The expression of HLA-DR is not decreased in these patients and it is plausible that an aberrantly high DR:DM ratio in B cells would facilitate the immunogenicity of DM-sensitive complexes. Recently, the murine homologue of HLA-DM has been shown to influence whether a T cell epitope is cryptic or immunodominant [23].

Our data show substantial variation in the DM susceptibility of different peptide/DR complexes at both neutral and endosomal pH. This concurs with earlier studies [24, 25]. While our experiments were performed using soluble DM, full-length DM from insect cells behaved similarly (data not shown). Thus, the variability we observed in the degree of DM enhancement of peptide release was not due to the lack of the transmembrane and cytoplasmic domains in soluble DM. Others have argued that DM susceptibility does not depend strongly on peptide sequence [26], in contrast to the implication of the results reported here. Follow-up studies are being performed to investigate the structural requirements for DM susceptibility.

These data imply that changes in the route of antigen presentation could result in autoimmunity. The 298–313 peptide presented on DR*0401 provides an example of a non-immunodominant complex that could be become immunogenic in cells in which HLA-DM activity is decreased. Similarly, the 322–337/DR4 complexes provide a first example of complexes that are stable at acidic but not neutral pH [27]. Interestingly, the pH of inflamed synovium may fall as low as 6.5 [28], and 322–337/DR*0401-specific T cells have been isolated from the peripheral blood of patients with RA [2, 29].

This study systematically relates peptide-MHC dissociation kinetics under different conditions to immunogenicity. It demonstrates that peptide/MHC complex kinetic stability throughout the antigen presentation pathway is a key requirement for immunodominance. In addition, it demonstrates how peptide/MHC complexes that are stable under some conditions but not others result in specific patterns of immune response that can include "peptide-only" T cells. Further study is required to determine if such peptide/MHC complexes, whose presentation could be altered by changes in endosomal pH, extracellular pH, or HLA-DM activity, play a role in autoimmunity.

4 Materials and methods

4.1 Complementary DNA constructs

Complementary DNA coding for the ectodomains of DRB1*0402 was isolated from the DR-homozygous EBV-transformed B cell line, AL10 by RT-PCR (Life Technologies; forward primer, 5'-CTGCTCGAATTCCCTGGTCCTGTCCT-GTTCTC-3'; reverse primer, 5'-GAAGAGCAGGTCGACC-TAGGTCTCTGGTTCAGGAGGTGGAGTCTTGCTCTGTGC-AGATTC-3'; the reverse primer attaches an epitope tag recognized by the mAb, KT3). The PCR product was cloned into the insect cell vector, pRmHA-3 [30], and constructs were verified by dye-terminator sequencing.

4.2 Cells

Schneider-2 (S2) *Drosophila melanogaster* cells were cultured in Schneider's Drosophila medium containing 10 % v/v FBS, 2 mM L-glutamine, and 50 μg/ml gentamycin. S2 cells expressing sDM (DMA*0101/DMB*0101) and HLA-DR*0401 (DRA*0101/DRB1*0401) have been described previously [31]. S2 cells expressing soluble DR*0402 were generated by co-transfecting S2 cells with pRmHA-3 containing a soluble DRA*0101 insert, DRB1*0402, and pUChs-Neo, using a calcium phosphate transfection kit (LTI). Cells were selected in 1.5 mg/ml active G418 (LTI), induced for 7 days with 1 mM CuSO$_4$, and expression was verified by Western blotting of tissue culture supernatants using an anti-DR antiserum (CHAMP; gift of L. Stern, MIT) and the epitope tag-specific

monoclonal antibody, KT3. Cells expressing sDR*0402 were cloned by limiting dilution in the presence of untransfected S2 feeder cells and 1.5 mg/ml G418. A clone with high expression was identified by Western blotting. For affinity purification, cultures were scaled up in 0.5L spinner flasks. Cells expressing sDM were adapted to growth in serum-free medium (BaculoGold Medium, PharMingen) before induction.

4.3 Purification of recombinant HLA-DR and DM molecules

sDM was purified by FLAG epitope tag affinity chromatography, followed by Sephacryl S200-HR size exclusion chromatography [32]. sDM protein was quantitated using a calculated extinction coefficient (79,270 $M^{-1}.cm^{-1}$). The protocol for immunoaffinity purification of recombinant DR molecules was similar to that previously described [33]. Eluates were analyzed for purity by SDS-PAGE; DRα and β chain bands comprised ≥85% of total protein. Heterodimeric assembly was checked by native PAGE, as described [32]. sDR protein was quantitated using the Bradford assay (Bio-Rad).

4.4 Synthesis and purification of fluoresceinated peptides

Synthesis of peptides (Research Genetics) was via FMOC solid phase synthesis on chlorotrityl resins (Novabiochem, La Jolla, CA) using diisopropylcarbodiimide/2-(1H-benzptriazole-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/N-hydroxybenzotriazole activation on Advanced Chemtech 396–5000 multiple peptide synthesizers (ACT, Louisville, KY). Protected amino acids (Sygena-Genzyme, Cambridge, MA) were double coupled at 8-fold excess for 1 h. The resin-peptide was resuspended in dimethylformamide (DMF) with a 5-fold excess of N-hydroxysuccinimidyl-fluoroscein and incubated overnight in the dark. This couples the fluorescein to the alpha amine group of the N-terminal amino acid. Resins were washed with dimethylformamide and methanol and peptides were cleaved in Reagent R [trifluoroacetic acid (TFA), ethanedithiol, thioanisole and anisole] [34]. The TFA mixture containing the peptide in solution was precipitated in ether and washed extensively. Preparative HPLC of peptides was accomplished by a gradient of 0–80% acetonitrile in 0.1% TFA. Lyophilization of the various fractions and verification by matrix-assisted laser description ionization time of flight (MALDI-TOF) using a Voyager mass spec (PerSeptive, Foster City, CA) yielded the synthetic peptides as a TFA salt. All peptide preparations used in this study were at least 90% pure.

The following N-terminal carboxyfluoresceinated peptides (f-peptides) representing immunodominant T cell epitopes defined in the HLA-DR*0401 transgenic mice were synthesized (the putative core epitopes are displayed in bold): f100–115 (NFGSQR**FSKIASNTQ**S), f262–276 (GRS**FTLAS-SETG**VGA), f322–337 (GNQWVG**YDDQESVKS**K), f327–337 (G**YDDQESVKS**K). Peptide 298–313 was synthesized with a

C to A substitution at position 300 to prevent peptide aggregation via thiol oxidation (EIADF**LRGATVHRT**LG); position 300 has been demonstrated to be irrelevant for T cell recognition by peptide truncation analysis (data not shown). Variants of 298–313, 299A, 302A and 303A, were made, in which residues [299]isoleucine, [302]phenylalanine and [303]leucine, respectively, were substituted with alanine.

4.5 Dissociation kinetics

Fluoresceinated peptide, 100 μM, was incubated with 2.5 μM soluble recombinant HLA-DR4 (DR*0401 or DR*0402), in a total volume of 50 μl, in PBS (150 mM sodium chloride, 10 mM sodium phosphate and 0.02% sodium azide) at pH 7.0 or in PBS acidified, usually to pH 5.5, with 100 mM sodium citrate. Following overnight (16–20 h) incubation at 37°C in siliconized microfuge tubes, the fluoresceinated-peptide/HLA-DR4 complex was separated from free fluoresceinated-peptide (f-peptide) at 4°C by size-exclusion chromatography, using a disposable chromatography column (Bio-Rad) packed with Sephadex G50-SF (Pharmacia), which had been preblocked with PBS/1% BSA at pH 7.0. The eluate (pH 7.0) was collected in a siliconized microfuge tube and acidified using sodium citrate, if required. sDM, 0.4μM, was added, where appropriate, immediately prior to measuring amount of complex at t=0. Unlabelled competitor peptide (10–100 μM) was added to all experiments. The influenza hemagglutinin peptide 306–318 was used as a competitor for all experiments with DR*0401 but was found to provide inadequate competition on DR*0402 ($t_{1/2}$ 4 h at pH 5.5 and $t_{1/2}$ 12 min at pH 5.5 in the presence of sDM). Unlabeled HCgp-39 peptide 298–313 was used as a competitor in experiments with DR*0402, since this peptide formed a more stable complex with DR*0402 than any of the others tested. All dissociation reactions were conducted at 37°C. The amount of initial f-peptide/DR4 complex was measured by taking a 15-μl aliquot from the total dissociation reaction volume of 260 μl at t=0, and analyzing it by high-performance size-exclusion chromatography, using a G3000SWXL TSK-GEL column (Tosohaas, Montgomeryville, PA) at room temperature, with a running buffer of 1 ml/min PBS pH 7.0. The chromatography column was connected to an in-line fluorescence detector (Gilson Fluorometer 121) set to deliver excitation at 495 nm and to measure emission at 525 nm. The fluoresceinated peptide/sDR peak eluted from the column at 9 min, followed by free peptide, which eluted between 11 and 17 min. Detection of the sDR heterodimer using absorbance of ultraviolet light at 280 nm indicated that most of the material was soluble and monomeric. Calibration of the f-peptide/sDR peak at t=0 (F0) against a 1-nM carboxyfluorescein standard indicated a concentration of f-peptide/DR between 9 and 24 nM. Since the 50-μl association reaction containing 2.5 μM DR had been diluted to a total volume of 260 μl after elution from the disposable Sephadex G50 column and approximately 50% of loaded sDR can be expected to elute from the Sephadex column (unpublished results), a theoreti-

cal maximum concentration of 240 nM f-peptide/DR complex is expected. The observed concentration represents 4–12% of this amount. The ratio of total sDM:total sDR is therefore approximately 2:1 and the sDM:f-peptide/DR complex is between 40:1 and 16:1. However, previous work with insect cell-derived full-length and sDM and sDR molecules have shown that soluble molecules exhibit approximately 200-fold lower activity than full-length DM [32]. The same study also indicated that this discrepancy was not due to misfolding of the sDM. Since Schafer et al. [35] have suggested that the DM:DR ratio in the endosomal compartment is approximately 1:5, we believe that our assay system uses a concentration of DM activity that is within the physiological range. The height of the initial peak representing the f-peptide/DR4 complex was designated F0 and peaks measured from subsequent time-points were displayed graphically as proportions of F0 (F/F0 ratio). The kinetics of f-peptide/DR4 dissociation were determined using single exponential curve-fitting functions in Kaleidagraph (Synergy Software), using the following equation: $[p/M]_t/[p/M]_0 = \exp(-k_{off}*t)$, where $[p/M]_t$ = concentration of f-peptide/sDR complex (M) at time t (s), $[p/M]_0$ = concentration of f-peptide/sDR complex (M) at time 0, k_{off} = dissociation constant (s^{-1}). The dissociation half-times ($t_{1/2}$) were derived using the equation: $t_{1/2} = \ln2/k_{off}$.

References

1 **Winchester, R.,** The molecular basis of susceptibility to rheumatoid arthritis. *Adv. Immunol.* 1994. **56:** 389–466.

2 **Cope, A. P., Patel, S. D., Hall, F., Congia, M., Hubers, H. A., Verheijden, G. F., Boots, A. M., Menon, R., Trucco, M., Rijnders, A. W. and Sonderstrup, G.,** T cell responses to a human cartilage autoantigen in the context of rheumatoid arthritis-associated and nonassociated HLA-DR4 alleles. *Arthritis Rheum.* 1999. **42:** 1497–1507.

3 **Buus, S., Sette, A., Colon, S. M., Miles, C. and Grey, H. M.,** The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science* 1987. **235:** 1353–1358.

4 **Muraro, P. A., Vergelli, M., Kalbus, M., Banks, D. E., Nagle, J. W., Tranquill, L. R., Nepom, G. T., Biddison, W. E., McFarland, H. F. and Martin, R.,** Immunodominance of a low-affinity major histocompatibility complex-binding myelin basic protein epitope (residues 111–129) in HLA-DR4 (B1*0401) subjects is associated with a restricted T cell receptor repertoire. *J. Clin. Invest.* 1997. **100:** 339–349.

5   **Vergelli, M., Kalbus, M., Rojo, S. C., Hemmer, B., Kalbacher, H., Tranquill, L., Beck, H., McFarland, H. F., De Mars, R., Long, E. O. and Martin, R.,** T cell response to myelin basic protein in the context of the multiple sclerosis-associated HLA-DR15 haplotype: peptide binding, immunodominance and effector functions of T cells. *J. Neuroimmunol.* 1997. **77:** 195–203.

6   **Chao, C. C., Sytwu, H. K., Chen, E. L., Toma, J. and McDevitt, H. O.,** The role of MHC class II molecules in susceptibility to type I diabetes: identification of peptide epitopes and characterization of the T cell repertoire. *Proc. Natl. Acad. Sci. USA* 1999. **96:** 9299–9304.

7   **Fairchild, P. J., Wildgoose, R., Atherton, E., Webb, S. and Wraith, D. C.,** An autoantigenic T cell epitope forms unstable complexes with class II MHC: a novel route for escape from tolerance induction. *Int. Immunol.* 1993. **5:** 1151–1158.

8   **Lee, C., Liang, M. N., Tate, K. M., Rabinowitz, J. D., Beeson, C., Jones, P. P. and McConnell, H. M.,** Evidence that the autoimmune antigen myelin basic protein (MBP) Ac1–9 binds towards one end of the major histocompatibility complex (MHC) cleft. *J. Exp. Med.* 1998. **187:** 1505–1516.

9   **Kasson, P. M., Rabinowitz, J. D., Schmitt, L., Davis, M. M. and McConnell, H. M.,** Kinetics of peptide binding to the class II MHC protein I-Ek. *Biochemistry* 2000. **39:** 1048–1058.

10 **Pinet, V., Vergelli, M., Martin, R., Bakke, O. and Long, E. O.,** Antigen presentation mediated by recycling of surface HLA-DR molecules. *Nature* 1995. **375:** 603–606.

11 **Santambrogio, L., Sato, A. K., Carven, G. J., Belyanskaya, S. L., Strominger, J. L. and Stern, L. J.,** Extracellular antigen processing and presentation by immature dendritic cells. *Proc. Natl. Acad. Sci. USA* 1999. **96:** 15056–15061.

12 **Arndt, S. O., Vogt, A. B., Markovic-Plese, S., Martin, R., Moldenhauer, G., Wolpl, A., Sun, Y., Schadendorf, D., Hämmerling, G. J. and Kropshofer, H.,** Functional HLA-DM on the surface of B cells and immature dendritic cells. *EMBO J.* 2000. **19:** 1241–1251.

13 **Pathak, S. S., Lich, J. D. and Blum, J. S.,** Cutting edge: editing of recycling class II:peptide complexes by HLA-DM. *J. Immunol.* 2001. **167:** 632–635.

14 **Rabinowitz, J. D., Tate, K., Lee, C., Beeson, C. and McConnell, H. M.,** Specific T cell recognition of kinetic isomers in the binding of peptide to class II major histocompatibility complex. *Proc. Natl. Acad. Sci. USA* 1997. **94:** 8702–8707.

15 **Iezzi, G., Karjalainen, K. and Lanzavecchia, A.,** The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* 1998. **8:** 89–95.

16 **Langenkamp, A., Messi, M., Lanzavecchia, A. and Sallusto, F.,** Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat. Immunol.* 2000. **1:** 311–316.

17 **Beeson, C., Rabinowitz, J., Tate, K., Gutgemann, I., Chien, Y. H., Jones, P. P., Davis, M. M. and McConnell, H. M.,** Early biochemical signals arise from low affinity TCR-ligand reactions at the cell-cell interface. *J. Exp. Med.* 1996. **184:** 777–782.

18 **Hammer, J., Gallazzi, F., Bono, E., Karr, R. W., Guenot, J., Valsasnini, P., Nagy, Z. A. and Sinigaglia, F.,** Peptide binding specificity of HLA-DR4 molecules: correlation with rheumatoid arthritis association. *J. Exp. Med.* 1995. **181:** 1847–1855.

19 **Watts, C.,** Capture and processing of exogenous antigens for presentation on MHC molecules. *Annu. Rev. Immunol.* 1997. **15:** 821–850.

20 **Rabinowitz, J. D., Vrljic, M., Kasson, P. M., Liang, M. N., Busch, R., Boniface, J. J., Davis, M. M. and McConnell, H. M.,** Formation of a highly peptide-receptive state of class II MHC. *Immunity* 1998. **9:** 699–709.

21 **Kropshofer, H., Vogt, A. B., Thery, C., Armandola, E. A., Li, B. C., Moldenhauer, G., Amigorena, S. and Hämmerling, G. J.,** A role for HLA-DO as a co-chaperone of HLA-DM in peptide loading of MHC class II molecules. *EMBO J.* 1998. **17:** 2971–2981.

22 **Louis-Plence, P., Kerlan-Candon, S., Morel, J., Combe, B., Clot, J., Pinet, V. and Eliaou, J.-F.,** The down-regulation of HLA-DM gene expression in rheumatoid arthritis is not related to their promoter polymorphism. *J. Immunol.* 2000. **165:** 4861–4869.

23 **Nanda, N. K. and Sant, A. J.,** DM determines the cryptic and immunodominant fate of T cell epitopes. *J. Exp. Med.* 2000. **192:** 781–788.

24 **van Ham, S. M., Grüneberg, U., Malcherek, G., Bröker, I., Melms, A. and Trowsdale, J.,** Human histocompatibility leukocyte antigen (HLA)-DM edits peptides presented by HLA-DR according to their ligand binding motifs. *J. Exp. Med.* 1996. **184:** 2019–2024.

25 **Vogt, A. B., Kropshofer, H., Moldenhauer, G. and Hämmerling, G. J.,** Kinetic analysis of peptide loading onto HLA-DR molecules mediated by HLA-DM. *Proc. Natl. Acad. Sci. USA* 1996. **93:** 9724–9729.

26 **Weber, D. A., Evavold, B. D. and Jensen, P. E.,** Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science* 1996. **274:** 618–620.

27 **Belmares, M. P., Rabinowitz, J. D., Liu, W., Mellins, E. D. and McConnell, H. M.,** pH stability of HLA-DR4 complexes with antigenic peptides. *Biochemistry* 2000. **39:** 14558–14566.

28 **Bodamyali, T., Stevens, C. R., Billingham, M. E., Ohta, S. and Blake, D. R.,** Influence of hypoxia in inflammatory synovitis. *Ann. Rheum. Dis.* 1998. **57:** 703–710.

29 **Verheijden, G. F. M., Rijinders, A. W. M., Bos, E., Coenen-de-Roo, C. J. J., van Staveren, C. J., Miltenburg, A. M. M., Meijerink, J. H., Elewaut, D., de Keyser, F., Veys, E. and Boots, A. M. H.,** Human cartilage glycoprotein-39 as a candidate autoantigen in rheumatoid arthritis. *Arthritis Rheum.* 1997. **40:** 1115–1125.

30 **Bunch, T. A., Grinblat, Y. and Goldstein, L. S.,** Characterization and use of the Drosophila metallothionein promoter in cultured *Drosophila melanogaster* cells. *Nucleic Acids Res.* 1988. **16:** 1043–1061.

31 **Sloan, V. S., Cameron, P., Porter, G., Gammon, M., Amaya, M., Mellins, E. and Zaller, D. M.,** Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature* 1995. **375:** 802–806.

32 **Busch, R., Reich, Z., Zaller, D. M., Sloan, V. and Mellins, E. D.,** Secondary structure composition and pH-dependent conformational changes of soluble recombinant HLA-DM. *J. Biol. Chem.* 1998. **273:** 27557–27564.

33 **Gorga, J. C., Horejsi, V., Johnson, D. R., Raghupathy, R. and Strominger, J. L.,** Purification and characterization of class II histocompatibility antigens from a homozygous human B cell line. *J. Biol. Chem.* 1987. **262:** 16087–16094.

34 **Albericio, F., Kneib-Cordonier, N., Biancalana, S., Gera, L., Masada, R. I., Hudson, D. and Barany, G.,** *J. Org. Chem.* 1990. **55:** 3730.

35 **Schafer, P. H., Green, J. M., Malapati, S., Gu, L. and Pierce, S. K.,** HLA-DM is present in one-fifth the amount of HLA-DR in the class II peptide-loading compartment where it associates with leupeptin-induced peptide (LIP)-HLA-DR complexes. *J. Immunol.* 1996. **157:** 5487–5495.

Correspondence: Grete Sonderstrup, Department of Microbiology and Immunology, Stanford University, Stanford, CA 94305, USA
Fax: +1-650-723-9180
e-mail: gretes@leland.stanford.edu