Pseudopeptide ligands for MHC II-restricted T cells

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Abstract

The potential therapeutic use of peptides to activate or anergize specific T cells is seriously limited by their susceptibility to proteolytic degradation. Classically, peptides are stabilized by incorporation of non-natural modifications including main chain modifications. In the case of MHC II-restricted peptides, the peptide backbone actively participates to the interaction with the MHC molecule and hence may preclude the peptidomimetic approach. We thus investigated whether a single amide bond modification influenced the peptide capacity to bind to a MHC II molecule and to stimulate specific T cells. Twenty pseudopeptide analogs of the I-E\(^d\) binder 24–36 peptide, whose sequence was derived from a snake neurotoxin, were obtained by replacing each amide bond of the peptide central part, by either a reduced ψ(CH\(_2\)–NH) or N-methylated ψ(CO–NMe) peptide bond. In agreement with the major interacting role played by the peptide backbone, several peptides displayed a low, if any, capacity to bind to the MHC II molecule and did not lead to T cell stimulation. However, one-third of the peptides were almost as active as the 24–36 peptide in I-E\(^d\) binding assays and one-fifth in T cell stimulation assays. Among them, two pseudopeptides displayed native-like activity. Good binders were not necessarily good at stimulating T cells, demonstrating that main chain modification also affected T cell recognition. We thus showed that a peptidomimetic approach could create a new type of MHC II ligand to control T cell responses.

Introduction

T cell peptides that efficiently bind to MHC molecules are considered as promising candidates to activate specific T cells and hence to provide new types of vaccines (1), especially against tumor cells (2). Paradoxically, they may also be interesting to prevent T cell stimulation and thus to make T cells tolerant to autoantigens (3,4) or allergens (5,6). In fact, T cell recognition of peptides embedded into the groove of MHC molecules does not necessarily lead to T cell activation but also to an anergic state or death. As inferred from in vitro experiments, a second signal arising from B7 engagement is required for the T cells to be activated (7,8). Otherwise, if only the TCR signal is provided to the T cells, they become insensitive to an external signal or die (9). Dendritic cells which display many B7 and MHC II molecules are considered as the main stimulating cells of naïve T lymphocytes (10), whereas peptide presentation by antigen-presenting cells (APC), devoid of co-stimulatory molecules or fixed by glutaraldehyde, leads to a tolerant state (11,12). As a result, in vivo injection of a T cell peptide or a protein mixed into an adjuvant recruits professional APC as dendritic cells and activates T cells, whereas the same antigen injected free in saline buffer provokes immune tolerance (13,14). Single amino acid substitutions in a T cell peptide determinant also create altered peptide ligands (APL) that induce a proliferative unresponsive state of the T cells, even in the presence of functional APC harboring appropriate co-stimulatory molecules (15). Most of these peptides seem to be recognized by TCR but induce unusual biochemical events inside the T cell (16,17). The potential use of APL to suppress pathogenic T cell expansion that occurs during autoimmune or allergic diseases is actually under investigation (18–20).

However, a major disadvantage of peptides is their sensitivity to proteases and hence their low lifetime in the physiologic fluids (21–23). The use of non-natural peptides to avoid proteolytic degradation is a logical extension of the peptide approach and has been widely used with success in various fields of medicinal chemistry, in particular by modifying the peptide backbone (23–25). Recently, attempts have been
made to introduce non-natural modifications into MHC class I and class II peptide ligands, especially on their side chains (26–28), whereas very few experiments were performed on the main chain (29–31). As revealed by crystallographic data, peptides bind to MHC II molecules in an extended conformation with both extremities protruding from the binding cleft (32–34). In agreement with these data, flanking regions generally tolerate various modifications without affecting the peptide binding capacity (35). Amidation and acetylation were found to favor the peptide resistance and its capacity to generally tolerate various modifications without affecting the main chain-modified T cell peptides.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequences</th>
<th>Molecular masses</th>
</tr>
</thead>
<tbody>
<tr>
<td>24–36</td>
<td>Ac C Y K V W R D H R G T I-NH₂</td>
<td>1773.8</td>
</tr>
<tr>
<td>N1</td>
<td>Ac C ψ[CO–NMe] Y K V W R D H R G T I-NH₂</td>
<td>1787.8</td>
</tr>
<tr>
<td>N2</td>
<td>Ac C Y K ψ[CO–NMe] K V W R D H R G T I-NH₂</td>
<td>1787.8</td>
</tr>
<tr>
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</tr>
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<td>N4</td>
<td>Ac C Y K ψ[CO–NMe] V W R D H R G T I-NH₂</td>
<td>1787.8</td>
</tr>
<tr>
<td>N5</td>
<td>Ac C Y K V ψ[CO–NMe] W R D H R G T I-NH₂</td>
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<tr>
<td>N6</td>
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</tr>
<tr>
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<td>Ac C Y K V W R ψ[CO–NMe] D H R G T I-NH₂</td>
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<tr>
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<tr>
<td>N10</td>
<td>Ac C Y K V W R D H ψ[CO–NMe] G T I-NH₂</td>
<td>1787.8</td>
</tr>
<tr>
<td>R1</td>
<td>Ac C ψ[CH₂–NH] Y K V W R D H R G T I-NH₂</td>
<td>1759.8</td>
</tr>
<tr>
<td>R2</td>
<td>Ac C Y ψ[CH₂–NH] K V W R D H R G T I-NH₂</td>
<td>1759.8</td>
</tr>
<tr>
<td>R3</td>
<td>Ac C Y K ψ[CH₂–NH] K V W D H R G T I-NH₂</td>
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<td>AR10</td>
<td>Ac C Y K V W R D H ψ[CH₂–NCOMe] G T I-NH₂</td>
<td>1801.8</td>
</tr>
</tbody>
</table>

Molecular mass was verified by mass spectroscopy.

Methods

Peptide synthesis

Protected amino acids, resins and reagents were from France Biochem (Meudon, France). Solvents were from SDS (Peypin, France). Metacresol and ethanedithiol were from Aldrich (St Quentin Fallavier, France). Peptides syntheses were carried out on an Applied Biosystem peptide synthesizer 430 A (Forster City, USA).

All the reduced bond peptides were synthesized by Boc strategy which is the only one compatible to reductive alkylation by LiAlH₄. Methylbenzylhydrylamine resin (0.57 mmol/g) was used to produce carboxamide C-terminus peptides. The different protecting groups were benzyl for threonine, cyclohexyl for aspartic acid, dinitrophenyl for histidine or benzoxymethyl for histidine aldehyde, 2-chlorobenzoxycarbonyl for lysine, 2-bromobenzoxycarbonyl for tyrosine, tosyl for arginine or dibenzoxycarbonyl for arginine aldehyde, formyl for tryptophan, and acetalidomethyl for cysteine. Aldehyde amino acids were prepared in two steps according to Fehrentz and Castro's method (40) without further purification. According to Sasaki and Coy's method, incorporation of ψ[CH₂–NH] isostere was usually complete within 1 h (41).

The N-methylated bond ψ[CO–NMe] was indifferently incorporated by either Fmoc or Boc strategy depending on
Fig. 1. Binding of main chain-modified peptides 24–36 to I-E\textsuperscript{d} measured in a competition assay. Fluoresceinated 24–36 (f24–36) was incubated with 10\textsuperscript{7} M immunoaffinity purified I-E\textsuperscript{d} molecule and different concentrations of reduced (A) or of N-methylated (B) bond peptides. After 48 h incubation, free peptides were separated from the complex by G50 and TSK columns. Inhibition percentage was calculated from fluorescence associated with the complex in the presence of competitor peptide relative to the fluorescence in the absence of competitor peptide. Results presented are from one experiment and deal with a limited number of analyzed peptides.

the availability of N-methylated and blocked amino acids. Boc protected histidine was N-methylated directly on the resin as previously described (41). On each side of the N-methylated linker, Boc-protected amino acids were incorporated in a double coupling reaction using PyBrop activation (43). The coupling reaction was monitored by the chloranyl test for secondary amines. Reduced and N-methylated peptides were acetylated with N-hydroxysuccinimide ester, cleaved by anhydrous hydrogen fluoride treatment with 2.5% ethanedi-thiol and 5% metacresol at 0°C for 60 min, and extracted into 10% acetic acid.

Fmoc strategy was used with analogs containing N-methylated valine, tryptophane, aspartic acid and glycine (Bachem, Voisins le Bretonneux, France). Syntheses were carried out on 4-[2',4'-dimetoxypfenyl-Fmoc-amino-methyl]-phenoxyresin (0.47 mmol/g) using DCC/HOBT coupling reagents. Side chains protecting groups were chosen as follows: 2,2,5,7,8-pentamethyl chroman-6-sulfonyl for arginine, tert-butyl for aspartic acid, threonine and tyrosine, acetamidomethyl for cysteine, tert-butylxycarbonyl for lysine and tryptophane, and trityl for histidine. As above, double coupling reactions by PyBrop activation were systematically required on each side of the N-methylated linker. Peptides were acetylated with acetic anhydride, deprotected and cleaved from the resin by 95% trifluoroacetic acid/2.5% H\textsubscript{2}O/2.5% trisisopropylsilane. Deprotected peptides were precipitated by cold diethylether and washed 3 times with ether.

After lyophilization, all the peptides were purified on semi-preparative reversed-phase HPLC Vydac C\textsubscript{18} column (250×20 mm) at 3 ml/min flow rate using acetonitrile/0.1% trifluoroacetic acid gradient (0–60% in 40 mn). Peptides were identified by electrospray mass spectroscopy and their homogeneity was assessed by analytical reversed-phase C\textsubscript{18} HPLC.

Peptide binding assay to I-E\textsuperscript{d} molecule
mAb 14-4-4S was purified from cell culture supernatants on a Protein A-Sepharose column as described by the manufacturer (Pharmacia, Saclay, France) and coupled on CNBr-Sepharose 4B column (10 mg antibody/5 ml gel). Large quantities (1–2×10\textsuperscript{15} cells) of A20.11.1 were cultured into DMEM (ATGC, Noisy le Grand, France) supplemented with 10% FCS, 4 mM glutamine, 2 mM sodium pyruvate, 100 µg/
Fig. 3. Relative positions of modifications introduced into the 24–36 sequence that lead to active pseudopeptides. The sequence of the 24–36 peptide (open circle) is given using one-letter amino acid code. Supposed TCR contact residues are indicated by upward arrows and I-E<sup>d</sup> contact residues are indicated by downward arrows. This assignment is based on our previous data (27) and the crystal structure (32). N-methylated and reduced bond peptides are placed at the position corresponding to their respective modification. Positions for which the modification caused an affinity change of the peptide toward I-E<sup>d</sup> lower than a factor of 10 have been retained and placed below the 24–36 peptide. Positions for which the corresponding peptide displayed a T cell activity change lower than a factor of 10 have been retained and placed above the 24–36 peptide.

Table 2. Binding to I-E<sup>d</sup> and T1C9 stimulating capacities of reduced and N-methylated bond analogs

<table>
<thead>
<tr>
<th>Peptides</th>
<th>I-E&lt;sup&gt;d&lt;/sup&gt; binding test</th>
<th>T cell hybridoma assay</th>
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<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td>Relative binding capacity</td>
</tr>
<tr>
<td>Ac24–36</td>
<td>2</td>
<td>–</td>
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<tr>
<td>N-methylated peptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>25</td>
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<tr>
<td>N2</td>
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<td>Reduced bond peptides</td>
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<tr>
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<td>&gt;100</td>
<td>&gt;50</td>
</tr>
<tr>
<td>AR10</td>
<td>2</td>
<td>1</td>
</tr>
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</table>

Binding to I-E<sup>d</sup> and T1C9 stimulating capacities were measured as described in Methods. IC<sub>50</sub> (concentration of competitor peptide leading to half intensity of fluorescence in binding assay) and ED<sub>50</sub> (effective dose of peptide leading to half maximum of T cell activation) were evaluated from at least two independent experiments. In both tests, the observed values did not differ by more than a factor of 3. Relative capacities were calculated using the 24–36 values as references.
fluoresceinated 24–36 (a kind gift from Dr G. Mourier) and incubated for 48 h at 37°C. It was subsequently loaded onto a small Sephadex G50 column (Pharmacia) and the eluate was analyzed by high performance size-exclusion chromatography with a Tosoh-Tas SSK SW3000 column (Touzard et Matignon, Courtaboeuf, France) connected to a fluorescence detector (Shimazu, Touzard et Matignon) and monitored by a LACHROM HPLC system (Merck, Nogent sur Marne, France). Fluorescence intensity was evaluated at 520 nm upon excitation at 494 nm, as the height of the peak associated with the MHC II molecule. Binding specificity was assessed by incubating the fluoresceinated peptides without MHC II molecules, and in the presence of both MHC II and a 100-fold excess of the non-labeled peptide. In both cases, no fluorescence was found at the retention time corresponding to the complexes (data not shown).

**T cell hybridoma stimulation assay**

A20 cells and T1C9 hybridoma (45) maintained in DMEM supplemented medium. T1C9 (5×10^6 cells) were plated into 96-well plates (Nunc, Polylabo, France) with A20 cells (5×10^4 cells) and serial dilutions of peptides in a final volume of 200 µl. Cultures were incubated 24 h at 37°C, and supernatants were subsequently harvested and frozen. Once thawed, the supernatants were subjected to CTLL assay. Briefly, IL-2-dependent CTLL were cultured in RPMI supplemented with 5% FCS, 2 mM glutamine, 10 mM HEPES, 100 µg/ml gentamicin and 5×10^−6 M 2-mercaptoethanol. After extensive washings, 10^5 cells were distributed into each well containing 50 µl of prewarmed supernatant. After 24 h incubation, [3H]thymidine (1 µCi/well, 5 Ci/mmol; Amersham, UK) was added. The cells were harvested 6 h later and filtered on microfiber filters. Incorporated thymidine was detected by scintillation counting using a β-counter (Wallac 1410, EG & G Berthold, Evry, France). Variations between experiments did not generally exceed 15%.

**Results and discussion**

Previously, we have delineated from region 24–36 of the Naja nigricollis neurotoxin α, a T cell epitope that binds to I-E^d^ and stimulates specific T cells, including a specific hybridoma called T1C9 (45). Using 24–36 analogs substituted by either L-alanine or D-amino acids, we intended to define peptide residues interacting with either the MHC molecule or the TCR (27). Our data suggested that region 25–33 is the T cell epitope core that binds to I-E^d^, and that Y25, R30 and R33 are the main MHC II anchor residues. By analogy with published structural data (32), we assumed that Y25 was buried into the P1 pocket which was well-known to accommodate aromatic or hydrophobic residues, while R30 and R33 interacted with the P6 and P9 pockets respectively. Basic residues at the C-terminal part of the peptide were previously designated as anchor residues for I-E^d^ by others (46,47) and found to take part in the E^d^ binding motif (48). It was also consistent with the recently published structure of I-E^d^ complexes in which the P9 pocket that was identical in the I-E^d^ molecule, accommodated a lysine (34). We used the 24–36 toxin α peptide to investigate the hydrogen bond contribution between the peptide backbone and the MHC II molecule. We thus systematically incorporated one chemical change at a time into all amide bonds between P1 to P10 residues as numbered by Stern et al. (32) and hence into a region encompassing the whole core epitope region (P1–P9) by one supplementary amino acid at each side. Two sets of peptides were synthesized. Since we previously showed that acetylation and amidation of the 24–36 peptide enhanced its T cell stimulating activity, all the peptides were acetylated and amidated (36). To investigate the role of carbonyl groups, we replaced the amide bond by a reduced peptide bond ψ[CH2–NH], whereas functionality of NH groups was probed by a N-methylated ψ[CO–NMe] replacement. One peptide called AR10 was found to have an unexpected acetylation of the NH and thus harbored two modifications of the same amide bond. Several milligrams of each peptide were purified to homogeneity and peptide identity was assessed by mass spectroscopy as indicated in Table 1.

**Influence of N-methylation and reduction on peptide binding capacity to I-E^d^**

First, the peptides were tested for their capacity to inhibit the binding of the fluoresceinated peptide 24–36 to I-E^d^, as illustrated in Fig. 1(A), broad differences between the reduced bond peptides were observed in their ability to compete with the labeled peptide. Only four peptides displayed binding capacity (R1, R2, R6 and AR10) whereas other analogs did not compete with the f24–36 peptide even at a maximal concentration of 10^{-6} M (Table 2). One peptide, AR10, bound to I-E^d^ as well as the native peptide with an IC50 ~ 2×10^{-6} M. In sharp contrast to reduced bond peptides, N-methylated peptides were all able to displace the fluoresceinated peptide (Fig. 1B and Table 2). In agreement with the binding capacity of the AR10 peptide that carried two modifications of the peptide backbone, peptide N10 demonstrated a similar behavior as 24–36. Interestingly, positions at which reduced bonds made the corresponding peptide still active (R1, R2 and R6) also tolerated well N-methylation (N1, N2 and N6). However, introduction of this backbone modification at other positions (N3, N4, N5, N7, N8 and N9) did not provoke a deleterious effect opposite to ψ[CH2–NH] but bound to I-E^d^ with IC50 values varying from 1.8 to 9.5×10^{-5} M.

It was not unexpected that some main chain-modified peptides exhibited substantial loss of binding toward I-E^d^. The crystal structures of HLAM-DR1 and HLA-DR3, complexed to HA 306–318 or CLIP peptide respectively (32,33), initially revealed the presence of an abundant network of hydrogen bonds between the peptide main chain atoms and HLA-DR. These interactions actively participate into the binding to MHC II molecules as illustrated by the good binding ability of polyalanine-based peptides, bearing only two anchoring side chains (29,37,38). Modification of the main anchoring points was therefore expected to dramatically affect these hydrogen bonds and hence the binding capacity of the peptide. Indeed, retro-inverso peptides in which the amide bond was reversed along the whole sequence weakly bind to MHC II molecules (29, 39). Conserved residues Ser953, Asn669, Asn882, Argx28, Trp861 and His881, that are present in all human and murine MHC II molecules, are implicated in these hydrogen bonds and seem to participate to an universal mode of peptide binding to MHC molecules (32,33) including murine
were still able to stimulate T cells. (29), several main chain-modified peptides retained substantial activity of MHC II molecules (34). Referring to these structural data, we assumed that R1, R3, R5, R6, R9, N1, N2, N6 and N9 have lost one hydrogen bond as compared to the native peptide with these residues. Clearly, R3, R5, R8, R9 and N9 analogs showed weak, if any, binding to I-E\textsuperscript{d}. In contrast, R1, N1, N2 and N6 peptides, in which modifications were also expected to eliminate one interaction with the MHC II molecule, displayed a relatively low decrease, suggesting a weak contribution from the corresponding hydrogen bonds to the binding to I-E\textsuperscript{d}. These observations are thus consistent with a differential contribution from the hydrogen bonds to stabilize peptide–MHC II complexes as suggested by similar experiments performed on HLA-DRB1*0101 and HLA-DRB1*0401 (29). As in our study, amide bond reduction appeared to affect the peptide binding more than N-methylation, which was never deleterious. Both types of modifications provoked varying effects related to the position in the peptide and to the HLA alleles, and did not necessarily correspond to those we observed on I-E\textsuperscript{d}. Thus, although the peptides bind to the MHC II molecule in an extended and common conformation, the peptide backbone seems to provide a number of contacts which contribute differently to binding from one combination of peptide–MHC II molecule to another. However, we should also consider that amide bond N-methylation or reduction modified the peptide flexibility and that by reducing the amide bond, protonation may occur as well as a tendency to adopt particular conformations (49). As a result, the binding capacity of the modified peptides could be indirectly disturbed. It should be the case for the R4 and R7 analogs which did not bind to I-E\textsuperscript{d} in our experiments, although the removed carbonyl was not expected to participate to the binding. However, regardless of the basis of all these effects, punctual backbone modifications did not necessarily lead to inactive peptides, as we previously observed for retro-inverso peptides which were modified along all the sequence (39). In agreement with others who made similar observations for HLA-DR molecules (29), several main chain-modified peptides retained substantial capacity to bind. The question arose as to whether they were still able to stimulate T cells.

**T cell stimulation by the N-methylated and reduced 24–36 peptides**

This question was addressed by means of a specific T cell hybridoma called T1C9 that secreted IL-2 upon presentation of the 24–36 peptide by I-E\textsuperscript{d}-bearing APC (44,36). As illustrated in Fig. 2(B) and Table 2, reduced bond peptides R3, R5, R6, R7 and R8 all failed to induce any detectable T cell stimulation even at a maximal concentration of 10\textsuperscript{-5} M, the native peptide 24–36 activating T1C9 at a mid-effective dose of 10\textsuperscript{-8} M. In sharp contrast to R6, which was previously characterized as a good binder, the lack of T cell reactivity from R3, R5, R7 and R8 was related to their previously observed failure to bind to I-E\textsuperscript{d}. On the contrary, analogs R1, R4, R9 and AR10 stimulated T1C9. In the case of R1, the loss of a carbonyl decreased T cell stimulation more than expected from the decreased binding to I-E\textsuperscript{d}, whereas R4 and R9 were T cell stimulating but displayed no ability to bind to I-E\textsuperscript{d}. A possible explanation for this finding is that the R4 and R9 peptides are in fact capable of binding to I-E\textsuperscript{d} at concentrations that are higher than those used in our assays. N-methylation of the amide bond appeared to affect T cell stimulation less than carbonyl reduction since all the N-methylated peptides activated T1C9. For most of the N-methylated peptides (N1, N2, N3, N4, N5, N7, N9 and N10), the T cell activity was diminished more than the MHC II binding capacity. However, as in the case of R4 and R9, the main chain modification in peptide N8 provoked an opposite effect. This peptide was only twice less active than 24–36 whereas its binding capacity was 30-fold less important than that of 24–36. One possibility was that the N8–I-E\textsuperscript{d} complex was a better ligand for T1C9 TCR than the 24–36–I-E\textsuperscript{d} complex. In agreement with the behavior of AR10, the peptide N10 that carried only one modification in its backbone also had a good T cell stimulating activity. Their activity did not greatly differ from that of the 24–36 peptide. The modified amide bond is located just after Arg33, which is assumed to be buried into P9 and thus should be located just at the end of the binding groove (27). It does not probably participate to contact with either the MHC II molecule or the TCR, justifying the native-like activity of both corresponding analogs. Peptide N6 was also a good T cell stimulating peptide but was modified in its central part. Interestingly, it competed efficiently with the fluoresceinated peptide for I-E\textsuperscript{d}, similarly to the R6 peptide. However, R6 did not stimulate T1C9, whereas it was modified on the carbonyl of the same amide bond as N6, located between Trp29 and Arg30. Thus, N6 formed 24–36-like complexes whereas R6 did not. Replacement of the carbonyl may directly affect T cell recognition or change the neighboring side chain orientation (48). Previously, we suggested that Arg30 was accommodated by P6, while Trp29 was at an ideal position to interact with the TCR (27). We observed that inversion of the configuration of Trp29 made the peptide unable to be recognized by 24–36-specific T cells, including T1C9, but still able to elicit specific T cells that were not in turn activated by the 24–36 peptide. Reduction of the amide bond might have modified the T cell reactivity by a similar mechanism. See Fig. 3.

**Concluding remarks**

MHC peptide binders are considered as interesting leads for immunotherapy. MHC class I-restricted peptides may be used to elicit cytotoxic T lymphocytes against viruses or tumors (1,2), while those restricted to MHC class II may be used to treat allergic or autoimmune diseases (3–6). In both cases, sensitivity of the peptides to proteolytic degradation is considered as a serious limitation that could be at least partially overcome by means of such non-natural modifications of the amide bond as reduction and N-methylation. Depending on the location of the modification in the peptide sequence, peptides with N-methylated and reduced bonds exhibited various effects toward either MHC II binding and T cell stimulation. Certain pseudopeptides did not display any binding activity whereas others especially N-methylated ones remained good binders. It suggests a differential contribution of the hydrogen bonds for the binding to the MHC II molecules (29). Moreover, these analogs also have interesting T cell reactivities as documented for the first time in this paper. N6 or N10 were good T cell stimulating peptides, while others, e.g. R6, were not. Then, as exemplified by N6 and N10,
pseudopeptides modified in adequate positions retained agonist activity. TCR antagonism effects were previously shown to result from subtle modifications of the side chains. As suggested by the behaviors of the N6 and R6 peptides, amide bond reduction could offer an alternative way to manipulate local conformation and/or neighboring side chain orientation. The possibility that main chain-modified peptides could also exhibit TCR antagonism remains to be addressed both in vitro and in vivo. To our knowledge, this study presents the first evidence on the use of peptidomimetics to control T cell responses.

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Abbreviations
APC antigen-presenting cell
APL altered peptide ligand
DM α-Dodecyl maltoside

References
2 Boon, T. and Van der Bruggen, P. 1996. Human tumor antigens
recognized by T lymphocytes. J. Exp. Med. 183:725.
3 Smilie, D. E., Wraith, D. C., Hodgkinson, S., Dwivedy, S., Steinman, L.,
and McDevitt, H. C. 1991. A single amino acid change in a
myelin basic protein peptide confers the capacity to prevent
rather induce experimental autoimmune encephalomyelitis. Proc.
Natl Acad. Sci. USA 88:9633.
experimental autoimmune encephalomyelitis and prevention of
relapses by treatment with a myelin basic protein peptide
analogue modified to form long-lived peptide–MHC complexes.
5 Hoyne, G., Bourne, T., Kristensen, N., Hetzel, C. and Lamb, J. R.
Immunopathol. 80:25.
6 Joost van Neerven, R. J., Ebner, C., Yssel, H., Kapsenberg, M.
L. and Lamb, J. R. 1996. T-cell responses to allergens: epitope-
7 Freeman, G. J., Freedman, A. S., Segil, J. M., Lee, G., Whitman,
J. F. and Nadler, L. M. 1989. B7, a new member of the Ig
superfamily with unique expression on activated and neoplastic
8 Freeman, G. J., Gribben, J. G., Boussiotis, V. A., Ng, J. W.,
Cloning of B7-2: a CTLA-4 counter receptor that costimulates
human T cell proliferation Science 262:909.
9 Schwartz, R. H. 1996. Models of T cell anergy: is there a common
11 Schwartz, R. H. 1990. A cell culture model for T lymphocyte clonal
12 Fuchs, E. J. and Matzinger, P. 1992. B cells turn off virgin but not
memory T cells. Science 258:1156.
13 Milich, D. R., Jones, J. E., McLachlan, A., Houghten, R., Thornton,
G. B. and Hughes, J. L. Distinction between immunogenicity and
tolerogenicity among HBcAg T cell determinants. J. Immunol.
143:3148.
induce in vivo tolerance selectively in IL-2 and IFN-γ-producing
15 Sloan-Lancaster, J., Evavold, B. D. and Allen, P. M. 1993. Induction
of T cell anergy by altered T-cell receptor ligand on live antigen-
Peptide major histocompatibility complex class II complexes with
mismatched agonist/antigen properties provide evidence for ligand-
related differences in T cell receptor-dependent intracellular
17 Rabinovitz, J. D., Beesson C., Wülfing, C., Tate, K., Allen, P. M.,
Davis, M. M. and McConnell, H. M. 1996. Altered T cell receptor
ligands trigger a subset of early T cell signal. Immunity 5:125.
18 Franco, A., Southwood, S., Arrenius, T., Kuchroo, V. K., Grey, H.
peptides are highly effective inhibitors of experimental allergic
19 Nicholson, L. B., Greer, J. M., Sobel, R. A., Lees, M. B. and
Kuchroo, V. K. 1995. An altered peptide ligand mediates immune
deviation and prevents autoimmune encephalomyelitis.
Immunity 3:397.
20 Tsitoura, D. C., Verhoeef, A., Gelder, C. M., O’Hehir, R. E. and
Lamb, J. R. 1996. Altered T cell ligands derived from a major
house dust mite allergen enhance IFN-γ but not IL-4 production
Peptide stability in drug development: a comparison of peptide
22 Powell, M. F., Stewart, T., Chvis, L., Urge, L., Grey, H., Gaeta, F.,
Sette, A., Arrenius, T., Thomson, D., Soda, K. and Colon, S. M.
1993. Peptide stability in drug development. II. Effect of single
amino acid substitution and glycosylation on peptide reactivity
of peptides and pseudo-peptides as a tool in peptide drug
24 Rodriguez, M., Lignon, M. F., Galas, M. C., Fulprand, P., Mendre,
biological activities of pseudopeptide analogues of the C-terminal
heptapeptide of cholecystokinin. On the importance of the peptide
25 Lebl, M., Sugg, E. E., Van Binst, G., Van der Elst, P., Pourve, D.,
Slaninova, J. and Hruby, V. J. 1987. Analogs of oxytocin containing
a modified peptide bond. Int. J. Peptide Protein Res. 30:318.
26 Alexander, J., Sidney, J., Southwood, S., Ruppert, J., Cserotto, C.,
Masel, A., Snoke, K., Serra, M. H., Kubo, S., Sette, A. and
Grey, H. W. 1994. Development of high potency universal DR-
restricted helper epitopes by modification of high affinity DR-
blocking peptides. Immunity 1:751.
27 Mailleìre, B., Mourier, G., Cotton, J., Hervé, M., Leroy, S. and
Menez, A. 1995. Probing immunogenicity by l-Ala and D-amino
design of nonnatural peptides as high affinity ligands for the
USA 92:753.
29 Hill, M. C., Liu, A., Marshall, K. W., Mayer, J., Jorgensen, B., Yuan,
B., Cubbon, R. M., Nichols, E. A., Wicker, L. S. and Rothbard,
J. B. 1994. Exploration of requirements for peptide binding to
30 Guichard, G., Calbo, S., Muller, S., Kurilsky, P., Briand, J. P. and
Abastado, J. P. 1995. Efficient binding of reduced peptide bond
pseudopeptides to major histocompatibility complex class I
31 Guichard, G., Connan, F., Graff, R., Ostankovitch, M., Muller, S.,
retro-inverse pseudopeptides as non-natural ligands for the human class I histocompatibility molecule HLA-A2. J. Med.
32 Stern, L. J., Brown, J. H., Jardetzky, T. S., Gorga, J. C., Urban,


