ArzneimForsch DrugRes

Sonderdruck / Reprint

Arzneimittel-Forschung
Drug Research

Editio Cantor Verlag · Aulendorf (Germany)
Arzneim.-Forsch./Drug Res. **47 (II)**, 9, 1069–1077 (1997)

af997S01

Biological Activity and Therapeutic Potential of Homologs of an Ii Peptide which Regulates Antigenic Peptide Binding to Cell Surface MHC Class II Molecules

Sharlene Adams[a, b], Fernando Albericio[c], Jordi Alsina[c], Emil R. Smith[b], and Robert E. Humphreys[a, b, d]

Antigen Express, Inc.[a], Worcester, MA (USA), Department of Pharmacology and Molecular Toxicology[b], and Medicine[d], University of Massachusetts Medical School, Worcester, MA (USA), and Department of Organic Chemistry[c], University of Barcelona, Barcelona (Spain)

Biological Activity and Therapeutic Potential of Homologs of an Ii Peptide which Regulates Antigenic Peptide Binding to Cell Surface MHC Class II Molecules

Sharlene Adams[a, b], Fernando Albericio[c], Jordi Alsina[c], Emil R. Smith[b], and Robert E. Humphreys[a, b, d]

Antigen Express, Inc.[a], Worcester, MA (USA), Department of Pharmacology and Molecular Toxicology[b], and Medicine[d], University of Massachusetts Medical School, Worcester, MA (USA), and Department of Organic Chemistry[c], University of Barcelona, Barcelona (Spain)

Summary

An invariant chain peptide (murine Ii76-92; Ii-Key) is known to produce a 10 to 50 times baseline enhancement of the presentation of specific antigenic peptides to murine T cell hybridomas by cell surface MHC class II molecules.

In order to define structure-activity relationships in Ii-Key, homologs were synthesized with the following systematic variations: 1) N- and C-terminal truncations, 2) N-terminal acetylation and C-terminal amidation, 3) substitutions with 13 natural amino acids in each position of the shortest, fully active peptide, and then with an additional 12 nonnatural amino acids at certain 'pharmacophore' positions, 4) substitutions with D-amino acids and N-methyl-leucine, and 5) cyclical forms. More than 160 homologs were tested for effects on antigen presentation by the murine MHC class II alleles: A^d, A^k, E^d or E^k. For some compounds, allele specificity between E^d and E^k exceeded 1 : 20. D-Amino acid and/or N-methyl-leucine substitutions were accepted at some residue positions, leading to peptides with relatively long half-lives in mouse serum and low toxicities in mice. An Ii-Key homolog inhibited in vitro presentation of an internally processed hen egg lysozyme determinant to a specific T hybridoma. The best compounds can be tested in vivo for therapeutic applications: 1) immunosuppression upon release of antigenic peptide, and 2) vaccination or immunomodulation upon co-administration of a second antigenic peptide.

Zusammenfassung

Biologische Aktivität und therapeutisches Potential von Homologen eines Ii-Peptides, welches die Antigen-Peptidbindung an Zelloberflächenmolekülen der MHC-Klasse II reguliert

Das invariante Kettenpeptid (Murin Ii76-92; Ii-Key) ist bekannt dafür, daß es die Wirkung der Zelloberflächenmoleküle der MHC-Klasse II, spezifische antigene Peptide den Murin-T-Zellen Hybridomas zu präsentieren, um das 10- bis 50fache erhöht.

Um die Struktur-Wirkungs-Beziehungen von Ii-Key zu untersuchen, wurden Homologe mit den folgenden systemischen Variationen synthetisiert: (1) Kürzung des N- und C-Terminus, (2) Acylierung des N-Terminus und Amidierung des C-Terminus, (3) Austausch der Aminosäuren des kleinsten aktiven Peptides mit 13 natürlichen Aminosäuren und anschließendem Austausch an pharmakophoren Positionen mit 12 unnatürlichen Aminosäuren, (4) Substitutionen mit D-Aminosäuren und N-Methyl-leucin und (5) auch mit zyklischen Resten. Untersucht wurde die Wirkung von mehr als 160 Homologe auf die Präsentation der Antigene durch die Allele der MHC-Verbindungen der Klasse II: A^d, A^k, E^d und E^k. Bei einigen Homologen war das Verhältnis der Allelen-Spezifität zwischen E^d und E^k größer als 1 : 20. Peptide, die mit D-Aminosäuren und/oder N-Methyl-leucin substituiert waren, zeigten teilweise eine relativ hohe Halbwertszeit im Serum von Mäusen und waren nur wenig toxisch. Eines der Ii-Key-Homologen hemmte in vitro die Präsentation eines intern verarbeiteten Hühnerei-Lysozyms „epitope". Die wirksamsten der synthetisierten Peptide können nun in vivo auf therapeutische Anwendungen getestet werden, wie (1) Immunosuppression nach Ausschüttung von antigenen Peptiden und (2) Impfung oder Immunmodulation, nachdem ein zweites, die Immunantwort förderndes, antigenes Peptid verabreicht wurde.

Key words Immunomodulation · Immunosuppression · Major histocompatibility complex class II · Peptide vaccine

Arzneim.-Forsch./Drug Res. **47 (II)**, 1069–1077 (1997)

1. Introduction

Many clinically important immune responses are initiated when antigenic peptides are presented to T cell receptors on $CD4^+$ T lymphocytes by MHC class II molecules on the surface of antigen presenting cells. Control of the selection of antigenic peptides, of the charging of antigenic peptides into the peptide-binding site of MHC class II molecules, or their release from that site, are all targets for drug design. Compounds regulating these processes have the potential either to enhance or to inhibit these selected immune responses.

The Ii protein plays an important immunoregulatory role in MHC class II functions. The Ii protein associates with MHC class II molecules at the time of synthesis and regulates the intracellular trafficking of MHC class

II molecules and the charging of antigenic peptides into the antigenic peptide binding site [1, 2]. Selection of antigenic epitopes is determined, in part, by the Ii protein, which blocks the antigenic peptide binding site of MHC class II molecules in the endoplasmic reticulum until it is cleaved and released in a specific post-Golgi antigenic peptide-charging compartment, allowing a recharging of the site with the ultimate antigenic peptide [2–4]. There the cleavage and release of Ii peptide appears to be regulated by two or three proteases: cathepsin B and D [4, 5] and perhaps cathepsin S [6]. The binding of a radioiodinated, azido function-crosslinking antigenic peptide to solubilized MHC class II molecules is enhanced during cathepsin B, but not cathepsin D, cleavage of Ii [7]. Nevertheless, the presence of trace levels of cathepsin D enhances cathepsin B generated increases in the rate of antigenic peptide binding to MHC class II molecules. Our finding of altered cathepsin B cleavage of the Ii peptide mutant hIi[$R^{78}\rightarrow A$, $K^{80}\rightarrow A$; $K^{83}\rightarrow A$; $K^{86}\rightarrow T$]* [8] indicated that one Ii segment which contains four clustered cationic-hydrophobic dipeptidyl units and which may contain cathepsin B cleavage sites is critical for the cleavage of MHC class II molecule-bound Ii protein. A peptide bracketing that segment in the Ii protein hIi77-92 (LRMKLPKPPKPVSKMR; substituting Y for the first L, as 'Ii-4', or now, 'Ii-Key') had been synthesized since it had six positive and no negative side chains. That sequence lead to the hypothesis that it might be a tightly linked, electrostatic signal to attract and dock a protease or other catalytic molecule to the Ii-MHC class II α,β trimer [9]. While a potent anti-Ii-4 heteroantibody was generated, it reacted only with denaturated Ii and the Ii peptide, and did not identify an external, native configuration of that sequence in Ii with MHC class II molecules. However, the Ii-Key peptide did greatly enhance the charging of antigenic peptides upon contacting cell surface MHC class II molecules in either mitomycin C-treated or paraformaldehyde-fixed cells [10]. The mechanism of action was found to be Ii-Key-mediated antigenic peptide exchange occurring on cell surface MHC class II molecules. As a step towards examining the therapeutic applications of this novel class of immunoregulatory compounds that act on cell surface MHC class II molecules, in the current study we have defined the structure-activity relationships for this peptide, and have developed backbone-protected, proteolysis-resistant homologs of relatively high activity and low toxicity.

2. Materials and methods

2.1. Antigenic and Ii peptides

The following antigenic peptides were used in the 'simultaneous assay' and in the 'antigenic peptide prepulse assay' or AE compound 'prepulse assay', respectively: HEL11–25, hen egg lysozyme 11–25, AMKRHGLDNYRGYSL (A^d); HEL46–61, hen egg lysozyme 46-61, NTDGSTDYGILQINSR (A^k); HEL106–116, hen egg lysozyme 106–116, NAWVAWRNRCK (E^d); PGCC81–104, pigeon cytochrome C 81–104, IFAGIKKKAERADLIAYLKQATAK (E^k); and THMCC82-103, tobacco hornworm moth cytochrome C 82–103, FAGLKKANERADLIAYLKQATK (E^k). The AE101 series peptides were obtained from the Tufts University Synthesis Facility or from Quality Controlled Biochemicals, Hopkinton, MA (USA), with the exception of the cyclical peptides. The cyclical peptides were synthesized in solid-phase, starting from an active carbonate resin according to a previously described procedure [11]. For both peptides, the first residue Fmoc-Lys-OAl was incorporated to the active resin through the side-chain in presence of a base (DIEA). For the head-to-tail cyclical peptide, elongation of the peptide chain was carried out using a standard Fmoc/tBu protocol [12]. After incorporation of the last residue, the allyl group was removed with Pd(0) and cyclization was carried out with BOP/HOAt/DIEA. Final cleavage of the anchoring linkage and side-chain protecting groups were performed with TFA in the presence of scavengers. The solution was filtered off, the peptide was precipitated with cold ether, centrifuged, washed with cold ether and centrifuged (this process was repeated four times) and lyophilized. For the side-to-tail cyclical peptide, the same procedure was used, but Fmoc-Lys(Alloc)-OH was used instead of Fmoc-Lys(Boc)-OH. At the end of the synthesis the last residue was acetylated. Removal of two allyl groups, cyclization and final cleavage was also performed as indicated above. In general, the purity and composition of each peptide was confirmed by HPLC and mass spectrometry. The native protein antigens (Sigma) were HEL-hen egg lysozyme, and PGCC, pigeon cytochrome C.

In all assays, antigenic peptides and the AE-101 series peptides were dissolved in phosphate-buffered saline (PBS; 0.01 mol/l sodium phosphate buffer, pH 7.2, 0.1 mol/l NaCl). The solutions were sterilized by filtration, and the peptide concentrations were determined by amino acid analysis (Applied Biosystems, 420A/130A derivatizer/HPLC after hydrolysis with 6 N HCl for 24 h in vacuo).

2.2. T cell hybridomas and antigen presenting cells

The following murine, MHC class II-restricted, antigenic peptide-specific T cell hybridomas were used. The TPci9.1 T hybridoma is specific for pigeon cytochrome C 81–104 peptide presented on the murine class II MHC allele E^k. The TPc9.1 hybridoma responds heteroclitically to tobacco hornworm moth cytochrome c 82–103 on E^k. The 3A9 T hybridoma is specific for hen egg lysozyme 46–61 on A^k. The 9.30.B2 hybridoma is specific for hen egg lysozyme 11–25 A^d, and the G28.C9 hybridoma is specific for hen egg lysozyme 106–116 on E^d. The A20 and CH27 B cell lymphoma lines, which express H-2^d and H-2^k alleles, respectively, were used as antigen presenting cells (APC).

2.3. The simultaneous assay

This asssay measures the ability of the AE101 series peptides to promote the exchange of endogenous, pre-bound peptides for specific antigenic peptides on cell surface MHC class II molecules [10]. In a simultaneous assay, 5×10^4 mitomycin C-treated APC, 5×10^4 T hybridoma cells (after 2200 rads) and a submaximal concentration of antigenic peptide were cultured with and without serial 4-fold dilutions of each AE101 series peptide, usually at 64, 16, 4, and 1 μmol/l, at PH 7.2–7.4, in complete DMEM-5 % FCS, 10 mmol/l HEPES, 1X nonessential amino acids (Sigma Chemical Co., St. Louis, MO, USA), 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 100 U/ml

---

Abbreviations

Al, allyl; Boc, tert-butyloxycarbonyl; BOP, benzotriazol-1-yl-oaxy-tris(dimethylamino-phosphonium hexafluorophosphate; DIEA, N,N-diisopropylethylamine; Fmoc, 9-fluorenylmethyloxycarbonyl; HEL, hen egg lysozyme; HOAt, 7-aza-1-hydroxybenzotriazole; Ii, invariant chain, MHC, major histocompatibility complex; PGCC, pigeon cytochrome C; tBu, ter.-butyl; TFA, trifluoroacetic acid; THMCC, tobacco hornworm moth cytochrome C.

The single letter amino acids codes used throughout all tables are as follows: A = L-alanine, Cit = L-citrulline, D = L-aspartate, E = L-glutamate, F = L-phenylalanine, H = L-histidine, Harg = L-homoarginine, K = L-lysine, k = D-lysine, L = L-leucine, l = D-leucine, mL = n-methyl-L-leucine, M = L-methionine, m = D-methionine, N = L-asparagine, Orn = L-ornithine, P = L-proline, p = D-proline, hydrP = L-hydroxyproline, R = L-arginine, r = D-arginine, Q = L-glutamine, Y = L-tyrosine, y = D-tyrosine, and W = L-tryptophan. Whenever mL appears in a table, it is set off by brackets to lessen confusion with 'd-methionine, L-Leucine'. Likewise, whenever Harg, Cit, Orn occur in a table, they are set off by spaces to lessen confusion, for example, with L-histidine, D-alanine, D-arginine, D-glycine' etc.

penicillin G, 100 μg/mL streptomycin sulfate, and 5×10^{-5} mol/l 2-mercaptoethanol (2-ME). Wells containing T + APC were included to monitor for background T cell activation; and wells containing T + APC + AE101 series peptide were included to monitor for non-specific T hybridoma activation by each AE101 series peptide in the absence of antigen. Similar wells receiving media in place of AE101 peptides provided the baseline level of antigen-specific T cell activation in the absence of the test compounds. Supernatants (aliquots of 20, 40 or 75 μl) from each culture were removed after 24 h and were assayed for the ability to support the growth of 1×10^4 interleukin-dependent HT-2 lymphoblastoid cells (added in 140, 120 or 75 μl complete Roswell Park Memorial Institute (RPMI) 1640 buffer – 10 % FCS, respectively), as measured by incorporations of [^{3}H]TdR, added at 1 μCi/well during the least 5 h of a 24 h HT-2 assay. For all assays, the reported value is the mean of triplicate wells, with a mean standard error of less than ± 10 %. Since the degree of cell stimulation and proliferation varied among assays, usually both in the primary T cell culture and in the secondary HT-2 indicator culture, for comparison among assays performed at different times, standard or reference peptides were always included. The data presented in this paper were obtained with usually 16 or 64 μmol/l of Ii-Key homolog in the primary culture and a 1 : 4 or 1 : 8 dilution of the culture supernate being taken into the HT-2 interleukin indicator assay.

2.4. The antigenic peptide release assay

This assay measures the ability of the AE101 series peptides to promote the release of the high-affinity antigenic peptides used as indicators in each system [10]. Antigenic peptide release was carried out under essentially the same conditions as described for the 'simultaneous assay' with the following modifications. Paraformaldehyde-fixed APC were first incubated for 6 h at 1×10^6 cells/ml in complete DMEM-5 % FCS in 24-well microculture plates (1 ml/well) with antigenic peptide, followed by four washes with 10 volumes of DMEM-5 % FCS. The cells were then exposed to varying concentrations of AE101 series peptide (64, 16, 4, and 1 μmol/l) for 24 h in the presence of the T cell hybridoma specific to the antigenic peptide. Interleukin release from these cultures was measured by proliferation of HT-2 cells to interleukins in supernatants transferred from the primary culture. Generally, a single dose of 64 μmol/l of each AE101 series peptide was used. The baseline T cell response was measured by culturing T hybridoma cells with the antigenic peptide-prepulsed APC in the absence of AE101 series peptides.

2.5. AE101 series peptide prepulse assay

This third assay measures the ability of the AE101 series peptides not only to promote the release of endogenous, pre-bound peptides but also to permit subsequent binding of specific antigenic peptides to cell surface MHC class II molecules [10]. This assay was carried out in the E^d and E^k systems as described for simultaneous assay with the following modifications. Fixed APC were first incubated for 6 h at 1×10^6 in DMEM-5 % FCS with 64 μmol/l of each AE101 series peptide or with PBS. The APC were then washed four times with ten volumes of DMEM-5 % FCS, and were cocultured with T hybridoma cells and submaximal doses of antigenic peptides used in the simultaneous assay. The baseline T cell response was measured by culturing T hybridoma cells with antigenic peptide and PBS-pretreated APC.

2.6. The processed antigen assay

This assay measures the ability of the AE101 series peptides to release antigenic peptides from peptide/MHC class II complexes that have formed within the APC as the result of natural antigen processing of the native protein. This assay was carried out under essentially the same conditions as the 'antigenic peptide release assay', with the following modifications. Untreated APC were incubated at 1×10^6/ml in 24-well plates (1 ml/well) with 10 μmol/l of native protein antigen (hen egg lysozyme, HEL) for 8 h. Following that incubation, the HEL-pulsed APC were washed, treated with mitomycin C, and washed again. AE101 series peptide was added at 64, 16, 4, and 1 μmol/l concentrations for 24 h in the presence of the T cell hybridoma specific for the synthetic antigenic peptide derived from the native protein. Interleukin release from these cultures was measured by proliferation of HT-2 cells to interleukins in supernatants transferred from the primary culture. The baseline T cell response was measured by culturing T hybridoma cells with the native HEL-prepulsed APC in the absence of AE101 series peptides.

2.7. Stability of peptides in mouse serum

All animal experiments were conducted on adult BALB/c mice (Taconic Farms, Germantown, NY, USA) and were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee. In a typical experiment, selected peptides were incubated at 250 μmol/l in fresh mouse serum at 37 °C for logarithmically increasing times, after which samples were iced and precipitated at 30 % trichloroacetic acid, and centrifuged. Aliquots of supernatants were separated by HPLC on a C18 reverse phase column using an acetonitrile : trifluoroacetic acid gradient in water. Retention times of each intact, pure peptide was determined under the same conditions in order to identify the relevant peak in the serum-incubated samples. Residual quantities of peptides were determined by interpolation of peak areas into a standard curve generated with dilutions of the intact, pure peptide. Loss of the peptide upon serum incubation was plotted as a function of time and half-lives were calculated. Molar extinction coefficients ranged from 1322 for AE235 at 276 nm to 9413 for AE324 (a p-nitrophenylalanine containing peptide) at 279 nm.

2.8. Acute toxicity of peptides

The minimum lethal dose for intravenous and intraperitoneal injections of two peptides was determined in male BALB/c mice injected with the peptides dissolved in sterile phosphate-buffered saline solution at doses of 10 mg/kg, 20 mg/kg, 30 mg/kg, and 100 mg/kg in 0.3 ml for intravenous injections, and the same doses plus a 1000 mg/kg dose in 0.6 ml for intraperitoneal injections. The mice were observed for at least 1 h immediately after injection and daily thereafter for 2 weeks, after which they were euthanized.

3. Results

3.1. Truncation homologs

In order to define the minimal, fully active peptide on the E^d and E^k MHC class II alleles, two respective series of truncations from the N-terminus and the C-terminus of murine Ii76-92 (full-length Ii-Key) were tested for the ability to promote peptide exchange on cell surface MHC class II molecules, as measured by enhancement of antigenic peptide presentation (Table 1). Ii-Key peptide activity decreased significantly upon deletion of three N-terminal residues (AE103, AE104, and AE105). In the 15 amino acid peptide AE102, removal of the first tyrosyl residue did not affect activity. Tyrosine had been subsituted for leucine in the native sequence in order to permit radioiodination of the peptide, and to increase the molar extinction coefficient, for the initial peptide AE101. Latter comparisons were made with homologs containing leucine (the wild type residue) rather than tyrosine.

Initial truncations from the C-terminus actually lead to increased activity as illustrated with AE108, in comparison to AE101, the parent peptide (Table 1). N-terminal acetylation and C-terminal amidation further increased activity (see AE109 and AE108), presumably due to inhibition of cleavage by aminopeptidases and carboxypeptidases, respectively. All subsequent peptide homologs were then examined as these terminally blocked forms. Although only a slight increase in activity was seen in multiple in vitro assays using terminally blocked, versus unblocked, peptides, it was expected that this protection would significantly increase biological half-life in vivo. Deletions of amino acids from the C-terminus of the 16 amino acid Ii-Key peptide lead to enhancement of activity, to a maximal value for the 7 amino acid peptide AE114 (E^d) and the 6 amino acid peptide AE115 (E^k). Acetylated and amidated AE114 then became a point of reference for all additional modifications. This peptide

Table 1: N- and C-terminal truncation homologs of AE101.

Peptide	Sequence	Simultaneous	
		E^d	E^k
None		1.0	1.0
AE101	YRMKLPKSAKPVSQMR	13.6	1.0
AE102	RMKLPKSAKPVSQMR	13.3	1.0
AE103	KLPKSAKPVSQMR	3.4	0.7
AE104	PKSAKPVSQMR	2.6	0.8
AE105	SAKPVSQMR	4.5	0.7
AE106	YRMKLPKSAKPVSQ	16.9	2.0
AE107	YRMKLPKSAKPV	21.7	1.0
AE108	YRMKLPKSAK	32.0	1.2
AE109	Ac-YRMKLPKSAK-NH_2	39.3	6.9
AE110	Ac-LRMKLPKSAK-NH_2	47.1	7.6
AE111	Ac-YRMKLPKSA-NH_2	39.2	7.2
AE112	Ac-YRMKLPKS-NH_2	42.8	15.3
AE113	Ac-YRMKLPK-NH_2	36.3	15.5
AE114	Ac-LRMKLPK-NH_2	39.8	15.9
AE115	Ac-YRMKLP-NH_2	19.9	18.6
AE116	Ac-YRMKL-NH_2	7.1	15.5
AE117	Ac-YRMK-NH_2	2.3	14.6
AE118	Ac-YRM-NH_2	1.0	5.6

AE114 (E^d) and AE115 (E^k) were the shortest, most active peptides in the truncation series. N- and C-terminally blocked peptides were more active than their un-blocked counterparts (AE109 vs. AE108, respectively). For these simultaneous assays, MHC class II-positive APC, treated with mitomycin C, were incubated for 24 h with antigenic peptide-specific T cell hybridomas, a submaximal dose of the respective antigenic peptide, and different concentrations of the AE101 series peptides. The concentrations of antigenic peptides used were 0.4 μmol/l of HEL106-116 for E^d and 0.075 μmol/l of THMCC82-103 for E^k. The data shown were generated using 64 μmol/l of each AE101 peptide. Interleukin released by the T hybridoma cells was quantitated by [^{3}H]TdR incorporation by interleukin dependent HT-2 cells using aliquots of culture supernatant. The values presented, 'Times Baseline Response', equaled the CPM of (T + APC + Ag peptide + AE101 series peptide)/ CPM of (T + APC + Ag peptide). The means of triplicate wells had an average SEM of ≤ 10 %. The T cell response to antigenic peptide alone ('None') was designated as the baseline value 1.0.

Table 2: Alanine scanning homologs of AE109 identify two major pharmacophore position.

Peptide	Sequence	Simultaneous	
		E^d	E^k
None		1.0	1.0
AE120	Ac-*A*RMKLPKSAK-NH_2	14.0	8.6
AE121	Ac-Y*A*MKLPKSAK-NH_2	*4.5*	*5.5*
AE122	Ac-YR*A*KLPKSAK-NH_2	11.0	12.2
AE123	Ac-YRM*A*LPKSAK-NH_2	13.9	13.8
AE124	Ac-YRMK*A*PKSAK-NH_2	9.3	18.2
AE125	Ac-YRMKL*A*KSAK-NH_2	*3.9*	*9.2*
AE126	Ac-YRMKLP*A*SAK-NH_2	8.6	20.4
AE127	Ac-YRMKLPK*A*AK-NH_2	9.3	16.9
AE109	Ac-YRMKLPKSAK-NH_2 (wild type)	15.4	15.2
AE128	Ac-YRMKLPKSA*A*-NH_2	15.0	13.7

Substituting alanine (A) for arginine (R) at the second position (AE121) and for proline (P) at the sixth position (AE125) in AE109 generated peptides with significantly decreased enhancement in a simultaneous assay as compared to the wild-type peptide, AE109. These two position define two pharmacophores, i.e., side chains which are critical for peptide activity. For simultaneous assay conditions, see Table 1. The data, 'Times Baseline Response', presented were generated using 16 μmol/l AE101 peptides for E^d and 64 μmol/l AE101 peptides for E^k. Substitutions are marked in italics. The AE101 series peptides were N- and C-terminally blocked.

contains the minimum sequences necessary for promoting antigenic peptide exchange on cell surface E^d and E^k MHC class II molecules, and has the identical amino acid sequence in human and murine Ii.

3.2. 'Pharmacophore' residues

An alanine scanning series was made from AE109 (10-mer) in order to define the residues which are critical for Ii-Key-mediated peptide exchange on I-E MHC class II molecules. Substitution of alanine at each residue position in AE109 defined the second position (arginine, AE121), and the sixth position (proline, AE125) to be particularly sensitive to activity loss (Table 2). Such positions with sensitivity to alanine replacement have been termed 'pharmacophores' and are primary targets for systematic replacement to determine structure-activity relationships. This analysis indicates that the N-terminal R X X X P motif contained in the AE101 series peptides, derived from the Ii sequence, plays a key role in interacting with I-E MHC class II molecules to promote the release of pre-bound peptides and to permit their replacement with specific antigenic peptides (enhancement of antigen presentation).

3.3. Amino acid replacements

In order to define the tolerances for different amino acid side chains at 6 of the 7 residue positions within the shortest, fully active Ii-Key peptide (AE114, 7-mer), a large series of 59 systematic, single amino acid substitution analogs was synthesized and tested on E^d and E^k. Substitution of 14 amino acids at each residue position 1, 2, 3, 4, 5, and 7 demonstrated general acceptance of all tested amino acids, other than those with negatively charged side chains, aspartic and glutamic acid. Results for substitutions at position 2 (Arg^{77}) and position 5 (Leu^{80}) are presented in Tables 3 and 4, respectively. In these studies, the most active amino acid was the naturally occurring amino acid or a close homolog, e.g., arginine and homoarginine at Arg^{77} (Table 3). In the position 2 substitution series (Arg^{77}), the simultaneous assay and the antigenic peptide release assay were completed in parallel. There is a 'mirror image concordance' between the Ii-Key peptide activities in each of these assays. That is, an amino acid substitution which had high activity in the simultaneous assay (peptide exchange),

Table 3: Homologs of AE109 with systematic substitutions at position 2 (Arg^{77}).

Peptide	Sequence	Simultaneous		Ag Release	
		E^d	E^k	E^d	E^k
None		1.0	1.0	1.0	1.0
AE109	Ac-YRMKLPKSAK-NH_2	13.3	2.2	0.28	0.28
AE121	Ac-Y*A*MKLPKSAK-NH_2	1.9	1.7	0.53	0.59
AE136	Ac-Y*E*MKLPKSAK-NH_2	3.4	1.1	0.54	0.97
AE135	Ac-Y*D*MKLPKSAK-NH_2	2.1	1.1	0.70	0.95
AE133	Ac-Y*H*MKLPKSAK-NH_2	3.2	1.2	0.39	0.86
AE138	Ac-Y*Q*MKKPKSAK-NH_2	1.5	1.7	0.73	0.85
AE140	Ac-Y*M*KLPKSAK-NH_2	1.6	1.4	0.78	0.79
AE137	Ac-Y*N*MKLPKSAK-NH_2	2.6	1.5	0.57	0.72
AE131	Ac-Y*Cit* MKLPKSAK-NH_2	9.3	1.9	0.69	0.71
AE130	Ac-Y*Orn* MKLPKSAK-NH_2	2.2	2.0	0.79	0.66
AE141	Ac-Y*M*KLPKSAK-NH_2	4.9	1.8	0.41	0.52
AE142	Ac-Y*L*MKLPKSAK-NH_2	8.8	0.82	0.86	0.46
AE134	Ac-Y*K*MKLPKSAK-NH_2	1.1	2.9	0.60	0.43
AE139	Ac-Y*F*MKLPKSAK-NH_2	2.4	1.9	0.67	0.38
AE132	Ac-Y *Harg* KLPKSAK-NH_2	8.8	4.4	0.58	0.01

On the E^d allele, the following four amino acids at the second position in AE109, where the wild-type amino acid is R, generated peptides with high activity: R, Cit, Harg, and L. On the E^k allele, the following three amino acids at position 2 in AE109 generated highly-active peptides: Harg, K, and Orn. The two replacements resulting in the least active peptides were D and E in both allelic systems. For simultaneous assay, see Table 1. The values presented for the simultaneous assay are the 'Times Baseline Response' generated using 16 μmol/l of AE101 series peptides for both alleles. Antigenic peptide release was carried out as described for the simultaneous assay (Table 1), with the following modifications. Paraformaldehyde-fixed APC were pre-pulsed with antigenic peptide by incubating for 6 h, followed by extensive washing and incubation for 24 h with T hybridomas and AE101 series peptides. For antigenic peptide release, the submaximal concentrations of antigenic peptide used were 3 μmol/l of HEL106–116 (E^d) and 20 μmol/l of THMCC82–103 (E^k). The 'Times Baseline Response' for antigenic peptide release was calculated as follows: (CPM of T + Ag pre-pulsed APC + AE101 series) / (CPM of T + Ag pre-pulsed APC). Substitutions are marked in italics. The AE101 series peptides were N- and C-terminally blocked.

Table 4: Homologs of AE114 substituted with tyrosine or with non-natural amino acids at position 5 (Lue^{80}).

Peptide	Sequence	Simultaneous	
		E^d	E^k
None		1.0	1.0
AE114	Ac-LRMKLPK-NH_2	47.0	2.8
AE235	Ac-LRMK *YPK-*NH_2	*51.0*	*26.8*
AE322	*Ac-LRMK(X2)PK-*NH_2	*37.0*	*3.0*
AE323	*Ac-LRMK(X3)*PK-NH_2	53.0	8.4
AE324	AcLRMK*(X4)*PK-NH_2	25.0	3.1
AE325	Ac-LRMK*(X5)*PK-NH_2	49.0	4.7
AE326	Ac-LRMK*(X6)*PK-NH_2	38.0	9.6
AE327	Ac-LRMK*(X8)*PK-NH_2	10.0	2.2
AE328	Ac-LRMK*(X9)*PK-NH_2	4.5	2.0
AE329	Ac-LRMK*(X12)*PK-NH_2	35.0	2.8
AE330	Ac-LRMK*(X13)*PK-NH_2	32.0	2.6
AE331	Ac-LRMK*(X14)*PK-NH_2	24.0	12.2
AE332	Ac-LRMK*(X15)*PK-NH_2	29.0	26.4

The most active replacement in position 5 of AE114 was with tyrosine (AE235). The higher activity of tyrosine (AE235) vs. phenylalanine (AE234, Table 5) is mimicked by the higher activity of Tic(OH), AE332, vs. Tic, AE331. The following side chain structures were substituted at position 5: X2 = p-chloro-Phe; X3 = p-fluoro-Phe; X4 = p-nitro-Phe; X5 = α-amino-4-phenylbutyrate; X6 = β-thienylalanine (Thi); X8 = di-bromo-tyrosine; X9 = di-iodo-tyrosine; X12 = β-1-naphthyl-alanine; X13 = β-2-naphthyl-alanine; X14 = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic); and X15 = 1,2,3,4-tetrahydroisoquinoline-7-hydroxy-3-carboxylic acid [Tic(OH)]. For simultaneous assay conditions, see Table 1. The value presented are the 'Times Baseline Response' generated using the following concentrations of AE101 series peptides: 4 μmol/l for E^d and 64 μmol/l for E^k. Substitutions are marked in italics. The AE101 series peptides were N- and C-terminally blocked.

was comparably high in the antigenic release assay. That pattern held in substitution series at each residue position.

3.4. Nonnatural amino acids at position 5, Leu^{80}

In order to define more clearly the putative size and structure of the pocket for the side chaim of Leu^{80}, peptides with 11 additional nonnatural amino acids or with tyrosine were tested on E^d and E^k (Table 4). The most active replacement was found to be with tyrosine (AE235) on both alleles. The higher activity of tyrosine vs. phenylalanine (Table 5) is mimicked in the higher activity of Tic(OH), AE332, vs. Tic, AE331. Tyrosine differs from phenylalanine and Tic(OH) differs from Tic by the presence of a distal hydroxyl group on a phenyl ring.

Table 5: Homologs of AE114 with systematic substitutions at position 5 (Leu^{80}).

Peptide	Sequence	Simultaneous	
		E^d	E^k
None		1.0	1.0
AE114	AcLRMK *L* PK-NH_2	10.6	10.7
AE225	Ac-LRMK *Orn* PK-NH_2	10.4	13.9
AE226	Ac-LRMK *Cit* PK-NH_2	9.0	9.0
AE227	Ac-LRMK *Harg* PK-NH_2	8.1	20.5
AE228	Ac-LRMK *H* PK-NH_2	8.5	20.3
AE229	Ac-LRMK *K* PK-NH_2	13.2	16.2
AE230	Ac-LRMK *D* PK-NH_2	0.6	1.1
AE231	Ac-LRMK *E* PK-NH_2	1.8	1.2
AE232	Ac-LRMK *N* PK-NH_2	12.5	8.9
AE233	Ac-LRMK *Q* PK-NH_2	11.5	17.0
AE234	Ac-LRMK *F* PK-NH_2	14.2	16.6
AE235	Ac-LRMK *Y* PK-NH_2	14.6	21.8
AE236	Ac-LRMK *M* PK-NH_2	15.2	16.9

In both the E^d and E^k systems, most substitutions were active as compared with AE114, excepting D and E. For simultaneous assay conditions, see Table 1. The values presented are the 'Times Baseline Response' generated using 64 μmol/l of AE101 series peptides. Substitutions are marked in italics. The AE101 series peptides were N- and C-terminally blocked.

Table 6: Activities of AE109 and the hydroxyproline^{81} homolog.

Peptide	Sequence	Simultaneous	
		E^d	E^k
None		1.0	1.0
AE109	Ac-YRMKL P KSAK-NH_2	16.4	4.3
AE143	Ac-YRMKL *hydrP* KSAK-NH_2	19.4	4.2

In both the E^d and E^k systems, replacing proline at the sixth position of AE109 with hydroxyproline (hydroP) generated a peptide with equal or greater activity. For simultaneous assay conditions, see Table 1. The values presented are the 'Times Baseline Response' generated using 64 μmol of the AE101 series peptides. Substitution are marked in italics. Both AE101 series peptides were N- and C-terminally blocked.

The Tic(OH) differs from tyrosine solely by the presence of a methylene (-CH_2-) bridge from the 2 position carbon on the phenyl side chain to the peptidyl imino group of the same residue. This concordance of activities in structurally homologous pairs of amino acids supports the view that a distal hydrogen bond is important to the I-E^k activity of these compounds. A similar pattern is not seen with I-E^d, in which that hydrogen bonding presumably does not occur. The size of the pocket in I-E^d molecules for the fifth position side chain can be estimated from the greater activity of the p-fluorophenylalanine (AE323) homolog than the p-chlorophenylalanine (AE322) homolog, and by the greater activity of the dibromotyrosine (AE328) homolog than the di-iodotyrosine (AE329) homolog. Structure-function activity relationships such as these will yield valuable information for designing allele-specific and allele-general, active Ii-Key-related drugs.

3.5. Proline and hydroxyproline at position 6

The sensitivity of Pro^{81} to replacement with alanine (Table 2) could reflect either a critical interaction of the prolyl side chain with the MHC class molecule or critical structure induced by the proline in either a cis or trans conformation. Since substitution with hydroxyproline did not significantly decrease activity (see AE143 versus AE109; Table 6), the requirement for proline is more likely to be due to an induced conformation than by interaction of the prolyl side chain with the MHC class II molecule.

3.6. Cyclical peptides

In order to test the effects of cyclization on Ii-Key peptide activity, two types of cyclical peptides were synthesized: 1) a 'head-to-tail', Leu^{76}-α-amino to Lys^{82}-carboxyl, 7 amino acid ring, AE381; and 2) a 'side-to-tail', Lys^{79}-ε-amino to Lys^{82}-carboxyl, 4 amino acid ring, AE382. Cyclization is a step towards synthesizing more restricted structures and is a classical step in drug design. The pattern of activity of the 7 amino acid ring differed distinctly from that of all other active, linear compounds in this study (Table 7). In the simultaneous assay in which enhancement indicates peptide exchange, AE381 was inhibitory (0.56, E^k) or barely active (2.1, E^d) in permitting charging with a second antigenic peptide compared to the linear parent peptide, AE114. In the antigenic peptide release assay, where inhibition indicates antigenic peptide release, AE381 was very active in releasing an already bound antigenic peptide (0.19, E^d and 0.28, E^k). Furthermore, in assays where the APC were first incubated with the AE compounds, AE381 inhibits on E^k and is inactive on E^d, thus indicating the subsequently added antigenic peptides cannot easily displace AE381, especially from E^k. These results were consistent with AE381 binding onto MHC class II molecules and displacing pre-bound, endogenous peptides,

Table 7: Contrasting activities of linear AE114 and two different cyclical homologs with identical amino acid sequences.

Peptide	Sequence	Simultaneous		Antigen release		AE101 prepulse	
		E^d	E^k	E^d	E^k	E^d	E^k
None		1.0	1.0	1.0	1.0	1.0	1.0
AE114	Ac-LRMKLPK-NH_2, linear	12.4	40.3	0.41	0.78	6.0	2.0
AE381	*L*RMKLP*K*, head-to-tail	2.1	0.56	0.19	0.28	1.0	0.3
AE382	Ac-LRM*K*LP*K*, side-to-tail	1.9	18.1	0.52	0.70	3.2	1.7
AE235	Ac-LRMKYPK-NH_2	12.5	56.6	0.30	0.80	2.1	15.6

AE381, the head-to-tail cyclical peptide, is inhibitory on E^k in all three assays, in contrast with the linear peptides: AE114 and AE235, which inhibit in only the antigen release assay. For the simultaneous assay (see Table 1) and for the antigen release assay (see Table 3), 64 μmol/l of AE101 series peptides was used for both alleles. In this antigen release assay, the antigenic peptide concentrations used during the prepulse were 24 μmol/l HEL106-116 for E^d and 1.25 μmol/l THMCC82-103 for E^k. The AE101 series prepulse assay was carried out as described for antigenic peptide release (Table 3), with the following modifications. Paraformaldehyde-fixed APC were incubated for 6 h with 64 μmol/l of each AE101 series peptide, followed by extensive washing and incubation for 24 h with T cell hybridomas and antigenic peptide, 0.3 μmol/l HEL106-116 for E^d and 0.4 μmol/l THMCC82-103 for E^k. For the AE101 series prepulse, the 'Times Baseline Response' was calculated as (CPM of T + Ag + AE101 series peptide pulsed APC) / (CPM of T + Ag + PBS sham pulsed APC). Residues involved in cyclization are marked with italics. The linear AE101 series peptides were N- and C-terminally blocked, and the side-to-tail cyclical AE101 peptide was N-terminally blocked.

but then failing to dissociate to permit a final 'locking in' of the subsequently added antigenic peptide. This apparently tightly binding property, the proteolysis resistance of such peptidyl rings, and the fact that ring-stabilized structures are amenable to modeling of organic scaffolds in directed drug design, makes AE381 a choice target for additional study as a immunosuppressant lead compound. The second cyclical peptide, AE382, behaved similarly to AE 114, the linear parent peptide; and did not exhibit the 'universal' blocking activity seen with AE381.

3.8. D-Amino acid and N-methyl amino acid peptides

Various D-amino acid (Table 8) and N-methyl amino acid (Table 9) homologs were tested to determine if these modifications would still result in active compounds. These substitutions are used to block proteolytic cleavage within the AE114 core Ii-Key peptide sequence.

Table 8. Certain homologs of AE114 substituted wiht single D-amino acids retain activity.

Peptide	Sequence	Simultaneous	
		E^d	E^k
None		1.0	1.0
AE114	Ac-LRMKLPK-NH_2	24.1	5.2
AE160	Ac-*l*RMKLPK-NH_2	11.0	1.7
AE161	Ac-L*r*MKLPK-NH_2	4.0	1.1
AE162	Ac-LR*m*KLPK-NH_2	2.2	1.3
AE163	Ac-LRM*k*LPK-NH_2	2.4	1.1
AE164	Ac-LRMK*l*PK-NH_2	17.7	3.8
AE165	Ac-LRMKL*p*K-NH_2	26.6	3.6
AE166	Ac-LRMKLP*k*-NH_2	26.4	3.4

Certain homologs with individual D-isomer amino acid substitutions retain biological activity: AE160, AE164, AE165 and AE166 for E^d and AE164, AE165 and AE166 for E^k. Lower case letters denote D-amino acid substitutions (italics). In both alleles, the C-terminal three positions tolerated single D-isomer substitutions better than the C-terminal portion of AE114. For simultaneous assay conditions, see Table 1. The values presented are the 'Times Baseline Response' generated using 64 μmol/l of the AE101 series peptides. The AE101 series peptides were N- and C-terminally blocked.

Table 9: AE114 Homologs with single N-methyl-leucine substitutions.

Peptide	Sequence	Simultaneous	
		E^d	E^k
None		1.0	1.0
AE114	Ac-LRMKLPK-NH_2	18	4.9
AE174	Ac-(*mL*)RMKLPK-NH_2	2	3.1
AE175	Ac-LRMK(*mL*)PK-NH_2	6	6.5

N-Methyl-leucine (*mL*) was accepted in positions 1 and 5 of AE114 with more loss of activity in the E^d system than in the E^k system. For simultaneous assay conditions, see Table 1. The values presented are the 'Times Baseline Response' generated using 4 μmol/l of the AE101 series peptides. See Table 10 for the effect of N-methyl-leucine substitution for methionine (M) in the third position. The AE101 series peptides were N- and C-terminally blocked.

Such modifications could be very valuable for prolonging the in vivo half-life of Ii-Key drug leads. D-amino acids were accepted in positions 5 (AE164), 6 (AE165), and 7 (AE166); and N-methyl-leucine was accepted in position 5 (AE175), without a great loss of activity. The retro-inverso, all D-amino acid homolog was completely inactive (data not shown). In the case of some peptide hormones, such retro-inverso D-amino acid homologs, in which the sequence is synthesized in reverse order with all D-amino acids, is active and completely protected against proteolysis. Several additional compounds, which were made to combine optimal characteristics of preferred side chains and backbone protection from proteolysis, maintained acceptable activity levels (Table 10).

3.9. Multiply substituted peptides to maximize activity and in vivo stability

Various peptides were synthesized incorporating combinations of D amino acids and/or N-methyl-leucines (Table 10) at positions in which the incorporation of

Table 10: In vitro serum half-lives of multiply substituted AE101 series peptides.

Peptide	Sequence	Half-life	Relative allelic activity	
			E^d	E^k
AE365	Ac-LR Harg *MY*PK-NH_2	23	5.2	135.4
AE235	Ac-LRMKYPK-NH_2	30	12.5	56.6
AE364	Ac-LRMKYP *Cit*-NH_2	30	12.2	111.8
AE368	Ac-LR*L*Kp*N*-NH_2	45	8.2	75.5
AE332	Ac-LRMK *(X15)* PK-NH_2	50	7.1	55.8
AE301	Ac-LR*L*KYPK-NH_2	55	12.5	59.9
AE307	Ac-LR*L*KYP*k*-NH_2	65	13.2	56.4
AE309	Ac-LR*L*K*W*PK-NH_2	70	12.1	48.2
AE305	Ac-LR*(mL)*KYP*k*-NH_2	130	5.2	26.8
AE308	Ac-LR*L*K*y*PK-NH_2	280	12.6	45.5
AE303	Ac-LR*(mL)*KYPK-NH_2	440	4.0	25.3
AE304	Ac-LR*(mL)*K*y*PK-NH_2	650	3.9	13.4
AE306	AcLR*(mL)*K*y*P*k*-NH_2	925	3.2	13.7

Certain multiply-substituted AE101 series peptides display both high activity and prolonged serum half-life. The AE235 peptide was the prototype used for the design of the other peptides listed. Substitutions are marked in italics. Lower case letter denote D-isomer amino acids, (mL) = N-methyl-leucine, Harg = homoarginine, Cit = citrulline; and X15 = 1,2,3,4-tetrahydroisoquinoline-7-hydroxy-3-carboxylic acid or Tic(OH), a non-natural amino acid. The peptides are listed in ascending order by half-life. The relative allelic activity is the 'Times Baseline Response' from data generated using 64 μmol/l of AE101 series peptides in simultaneous assays (see Table 1 for conditions). The half-life (min) was determined by incubating a known concentration of the AE101 series peptide in fresh mouse serum at 37 °C. Aliquots were removed at different time points, and serum proteins were precipitated using trifluoracetic acid (30 % final volume), followed by centrifugation. HPLC was used to quantitate the remaining intact AE101 peptide, comparing the area under the peptide peak with a standard curve generated using samples containing known concentrations intact peptide. The AE101 series peptide were N- and C-terminally blocked.

single residues of these amino acids did not have more than 50 % loss of activity (Tables 8 and 9). These multiply-substituted peptides were designed and synthesized as a first step towards future in vivo experiments. The activities of these compounds in the simultaneous assay on E^d and E^k alleles were determined. The half-life for loss of the peptide due to proteolysis in mouse serum at 37 °C was also presented for each peptide. Among these compounds, increasing the degree of backbone protection from proteolysis correlated with increased serum half-life. Readily finding such compounds indicates that yet additional active, protected compounds can be designed for future studies.

3.10. Stability of peptides in mouse serum

Since it was anticipated that the peptides being studied would be degraded by serum peptidases and that this might be an important determinant of their biological half-life, with resistance to degradation being advantageous, the stability in mouse serum of 13 peptides was measured and compared to their relative allelic activities (Table 10). The 13 compounds exhibited a wide range of stability in serum, with half-lives of degradation ranging from 23 to 925 min, and a wide range of relative allelic activities. Importantly, compounds with very long serum half-lives, like AE304 and AE306, exhibited at least some biological activity, and one compound, AE308, exhibited both a relatively long serum half-life and potent biological effects. Thus, among these compounds the structure requirements for stability in serum do not preclude biological potency.

3.11. Acute toxicity of peptides

Preliminary, dose-ranging acute toxicity studies were conducted on AE114 and AE235 to assess the relative lethality of these compounds and, if possible, to identify target organs for their toxic effects and, perhaps, other members of this group. Mice were given single, rapid i.v. injections of 10, 20, 30, or 100 mg/kg of AE114 or of AE235 (1 mouse/dose/peptide) in a volume of 300 µl of PBS for each dose. These doses of AE114 and AE235, plus 1000 mg/kg for AE235, were given as single i.p. injections (1 mouse/dose/peptide) in a volume of 600 µl of PBS for each dose.

Intravenous administration of 10 mg/kg was without effect for both peptides tested, while 30 mg/kg of AE114 and 20 mg/kg of AE235 immediately produced transient convulsions and gasping. Regression of these symptoms was followed by a more persistent mild sedation. The mice recovered fully within 2 h. In contrast, i.v. doses of 30 mg/kg and above of AE235 and 100 mg/kg of AE114 were lethal, with tachypnea, exophthalmus and transient convulsions rapidly leading to death within 1–2 min. Examination of the heart at death revealed ventricular arrest with atrial flutter or fibrillation. Upon i.p. administration, doses from 10 to 30 mg/kg of either peptide were without discernible effect, while 100 mg/kg of AE114 or AE235 produced a slowly developing and reversible sedation. The 1000 mg/kg dose of AE235 was lethal, with rapid onset of sedation (5 min), followed by convulsions and gasping until death occurred at 30 min post-injection.

3.12. In vitro suppression of presentation of processed protein antigen

The presentation of a hen egg lysozyme determinant by E^d to a specific T hybridoma after uptake and processing of 10 µmol/l hen egg lysozyme, was inhibited by AE114, AE235, the head-to-tail cyclical peptide, AE381, and side-to-tail cyclical peptide AE382 (Table 11) in an assay similar to antigenic peptide release. Comparable results were obtained with a tetanus toxoid specific human T cell line with respect to tetanus toxoid presented by an autologous EBV-transformed line (Dr. Quoc V. Nguyen, personal communication). These experiments demonstrate the efficacy of these compounds in suppressing the immune response in vitro and support further in vivo trials with compounds with good pharmacokinetic and toxicological properties.

Table 11: Presentation of antigenic peptide generated through processing of native antigen by APC is inhibited by AE101 series peptides.

Peptide	Sequence	E^d
NOne		1.0
AE114	Ac-LRMKLPK-NH_2	0.22
AE235	Ac-LMKYPK-NH_2	0.19
Ae381	*L*RMKLP*K*, head-tail cyclical	0.18
AE382	Ac-LRMKLP*K*, side-top-tail cyclical	0.61

Both linear and cyclical AE101 series peptides inhibit the presentation of antigenic peptides, which are generated by processing within APC. The processed antigen assay was carried out as described for antigenic peptide release (Table 3), with the following modifications. APC were incubated for 6 h with 10 µmol/l of native HEL, followed by extensive washing and treatment with mitomycin C. The HEL-pulsed APC were incubated for 24 h with T cell hybridomas and 64 µmol/l of AE101 series peptides. The 'Times Baseline Response' was calculated as follows: (CPM or T + HEL-pulsed APC + AE101 series peptide) / (CPM of T + HEL-pulsed APC). The AE101 series peptides were N- and C-terminally blocked.

3.13. N-terminal CLIP truncation homologs

In contrast to the enhancing effects of Ii-Key homologs on antigenic peptide presentation to specific T cell hybridomas in simultaneous assays, Ii80-103 (full-length CLIP) was shown to competitively inhibit antigenic peptide presentation [10]. Since the C-terminus of full-length Ii-Key (Ii76-92) overlaps the N-terminus of the full-length CLIP (Ii80-103), a series of N-terminal truncation analogs of Ii80–103 was synthesized and tested to determine the minimum CLIP sequence necessary to be inhibitory in the murine T cell assays. The active region for this blocking effect, as demonstrated with a series of N-terminal truncations of CLIP (Table 12) in these murine antigen presentation assays, was to the N-terminus of R^{91}, from K^{85} to M^{90}. The LPK motif at the N-terminus of Ii80-103 (long CLIP) is the C-terminal three amino acids of the minimum Ii-Key 7-mer: LRMKLPK (AE114). Since Ii85-103 and Ii87-103 are still potent inhibitors, even though they share no residues with AE114, it appears that the core Ii-Key sequence and the CLIP motif for blocking antigen presentation can be se-

Table 12: N-terminal truncation homologs of murine CLIP (mIi80-103) separate the active CLIP class II-blocking region from the Ii-Key core sequence.

Peptide	Sequence	Simultaneous	
		A^d	A^k
None		1.0	1.0
mIi80-103	*LPK*SAKPVSQMRMATPLLMRPMSM	0.2	0.01
mIi85-103	KPVSQMRMATPLLMRPMSM	0.06	0.04
mIi97-103	VSQMRMATPLLMRPMSM	0.26	0.04
mIi90-103	QMRMATPLLMRPMSM	0.82	0.36
mIi91-103	RMATPLLMRPMSM	0.96	1.0
mIi92-103	MATPLLMRPMSM	0.96	1.8
mIi93-103	ATPLLMRPMSM	1.0	1.3

The N-terminal three residues of mIi80-103 ('full-length murine CLIP') are the C-terminal three residues of AE114, the minimal Ii-Key peptide (italics). Simultaneous assays were carried out as described in Table 1, using paraformaldehyde-fixed APC. The concentrations of antigenic peptides used were 0.05 µmol/l of HEL11-25 (A^d) and 0.05 µmol/l of HEL46-61 (A^k). The values presented are the 'Times Baseline Response' generated with 64 µmol/l of the CLIP-region peptides. The CLIP-region peptides were not N- and C-terminally blocked.

parated. This suggests there could be two distinct regulatory sites on MHC class II: the antigenic peptide-binding groove, to which CLIP binds, and one other site, to which the core of Ii-Key binds.

4. Discussion

These studies define the active core of the 16 amino acid peptide, murine Ii(77-92), to be the N-terminal, 7 amino acid segment LRMKLPK. The 16-mer (then Ii-4, now Ii-Key) was originally found to enhance the presentation of antigenic peptides to T cell hybridomas by promoting peptide exchange on cell surface MHC class II molecules [10]. Within the 7 amino acid segment, an 'alanine scan' demonstrated that the second residue, R^{77}, and the sixth residue, P^{81}, were particularly important for biological activity. Such alanine-sensitive positions have been termed 'pharmacophores' and are usually the first targets for systemic replacement with various natural and nonnatural amino acids to define structure/activity relationships. We extended such studies to the other residue positions with 11 amino acid replacements, excepting the fifth position with 28 amino acids and the sixth position with 2 (alanine and hydroxyproline). Notably, a wider range of replacements than might be expected were accepted in all positions. That is, most substitutions, excepting the negatively charged side chains Asp and Glu, generally yielded biologically active homologs. There was a reproducible variation in activities among homologs with amino acid replacements at any one residue position in terms of activities for E^d vs. E^k alleles. This finding at first led us to target modifications in order to achieve significant degrees of allele specificity, in a first step towards preferentially disrupting antigen presentation at alleles associated with the induction or progression of MHC class II-associated autoimmune diseases. However, throughout this study the maximal degree of allele specificity ($E^d : E^k$) that could be obtained was generally below 1 : 4 or 4 : 1, with a few exceptions approaching 9 : 1 or 1 : 24. That observation and the expectation of few allele-specific, side chain replacements in the MHC class II allosteric site of action of the Ii-Key compounds (outside the antigenic peptide binding groove, where the high degree of polymorphism in MHC class II molecules is observed) now leads us to believe that a clinically useful degree of MHC class II allele specificity for these compounds might not be obtained. It is not unusual that some degree of allele specificity is observed with these relatively small peptides, however, in spite of the fact that the intact Ii protein binds well to all MHC class II molecules. In contrast to the relatively weak allele specificity of individual compounds, there was a notable MHC class II locus specificity for the Ii-Key peptides as a group. While these compounds were highly active on E^d and E^k, they were virtually inactive on A^d and A^k, at least as tested with the antigenic peptides and hybridomas used in this study. If such a locus specificity also occurs in comparable studies with human MHC class II molecules, then locus specific drugs might be created.

The first objective of our structure-activity relationship (SAR) analysis was to identify compounds with optimal potency and protection against proteolysis, as a step towards in vivo testing. Abstractly in drug design, one might sacrifice potency in a purified receptor assay (or in vitro test assay) in favor of protection against proteolysis or some other pharmacokinetic characteristic which makes for a more useful drug. Some, but still acceptable, activity was lost with certain modifications which did offer substantial protection against protease inactivation. N-terminal acetylation and C-terminal amidation, which protect against the respective exopeptidases, actually increased activity reproducibly in the in vitro assays. In some residue positions, D-amino acids were accepted with only modest loss of activity, although unacceptable loss occurred with individual replacement in the first through fourth residue positions of AE114. N-methylleucine was accepted in the third position, replacing methionine, and in the fifth position, replacing leucine. A series of peptides with multiple D and/or N-methyl amino acid replacement had significantly prolonged half-lives in murine serum at 37 °C. Those final compounds of this study, with good proteolysis resistance in serum, were relatively nontoxic. That low toxicity will permit immediate study of therapeutic applications in animal models, while the search continues for homologs best suited for human therapies.

The real question is: what happens in an animal when antigen can be introduced locally or systemically into cell surface MHC class II molecules without the classical effects of adjuvants? Classical adjuvants have two properties: 1) APC activation, or activation of cells around the APC, and 2) the physical property of prolonged release of antigen at significant local concentrations. Here we have a method to enhance the binding of antigenic peptides, and structures designed to induce immunomodulation, without inducing the adjuvant effects which favor a Th1 response. While these experiments address first the development of novel therapeutic compounds and methods to present antigenic determinants freshly bound by cell surface MHC class II molecules to T cells, they also do contribute to an understanding of the mechanisms regulating intracellular charging of antigenic peptide to MHC class II molecules.

Toward understanding the molecular mechanism of these compounds, let us first consider possible models for MHC class II binding of the Ii-Key compounds based on crystallographic images of an antigenic peptide and of a CLIP segment binding to HLA-DR3. Close overlap in binding of influenza virus hemagglutinin peptide HA307-319 and an Ii-CLIP peptide in HLA-DR3 was defined by x-ray crytallography [13, 14]. In both cases, the peptides assumed the conformation of a polyprolyl type II helix in the antigenic peptide binding groove. The backbone atoms of the CLIP peptide overlay almost exactly the positions of the backbone atoms of the HA peptide, with comparable placement of side chains into pockets of the MHC class II molecule. Residue position M^{91} of the CLIP peptide was found to be anchored in the first deep 'pocket' in the antigenic peptide-binding groove of DR3. The CLIP residues N-terminal to M^{91}, extending back to P^{87}, were also in a polyprolyl type II helix conformation. More N-terminal residues, including positions human Ii L^{77}-K^{83} which correspond to the AE114 compound, were not resolved in those crystallographic studies, but would clearly lie outside the antigenic peptide binding groove, along the 'side' of the MHC class II molecule. Thus, the putative site for activity of the AE114 compound (Ac-LRMKLPK-NH_2) lies outside the antigenic peptide binding site if the Ii-Key peptide lies in registry with the alignment of the CLIP peptide. The AE114 site would therefore be considered to be allosteric with respect to binding and release of antigenic peptides at the antigenic peptide binding groove, since it is at a second position on the same molecular complex but exerts an effect at the first position – the antigenic peptide binding site. Our observations that mIi85-103 is a potent inhibitor of antigen presentation while mIi76-82 (AE114) is sufficient to promote antigenic peptide release and recharging, supports this view of two physically distinct sites of action of these Ii sequences.

Although the above model for the active site seating of AE114 homologs places that site at one end of and outside the antiparallel alpha helices which hold the anti-

genic peptides in MHC class II molecules, additional experimental data of others also support this view. Urban and Strominger observed that the lability of CLIP homologs at pH 4.0 depended on the N-terminal amino acid residues from human Leu^{81}-Ser^{89} (LPKPPKPVS) [15]. Kropshofer et al. [16] determined that at an endosomal pH 5.8, the relatively rapid dissociation of Ii (81-105), a 'long CLIP' peptide from HLA-DR3, depended on the presence of the identical N-terminal segment human Leu^{81} to Ser^{89}. In studies of an 'alanine scanning' series of CLIP peptides, with alanine substitutions at each of those residue positions, the individual alterations eliminating the rapid dissociation of CLIP were at Lys^{83} and Pro^{87}. The alanine scanning series of Ii-Key peptides presented here identified the same motif: K X X X P, to be critical for Ii-Key-mediated peptide exchange on cell surface MHC class II molecules.

A most intriguing AE101 series compound is the head-to-tail cyclical peptide, AE381, which has the same amino acid sequence as the linear AE114. AE381 inhibited antigenic peptide presentation to T cells in three different types of functional assays on E^k (simultaneous, antigenic peptide release, and AE101 peptide prepulse), and only weakly enhanced in simultaneous and AE101 peptide prepulse assays on E^d. In contrast, AE114 and the related homolog, AE235, enhanced peptide presentation to T cells in the simultaneous and AE101 prepulse assays and inhibited only in the antigenic peptide release assay. Both AE114 and AE235 promote peptide exchange on cell surface MHC class II molecules. AE381, however, promoted the release of pre-bound peptides but did not permit the binding of a second peptide (no exchange). Out of the over 160 AE101 series homologs tested thus far, no other peptide behaved in the same manner as AE381. The only other Ii peptides known to inhibit in these three assays are mIi80-103 and hIi81-104, ('full length' murine and human CLIP, respectively) [10]. While the CLIP-region peptides inhibit across these three assays by 1) promoting the release of pre-bound peptides and 2) binding to and blocking the antigenic peptide binding groove, AE381 must inhibit through a different mechanism because it lacks the groove-binding sequence found in the CLIP-region peptides. The working hypothesis is that the AE381 cyclical peptide binds to the allosteric site on E^k and release pre-bound peptides, but then does not dissociate readily, leaving the MHC class II molecules 'frozen' in an destabilized conformation that cannot bind antigenic peptides. Small, stable, organic compounds which mimic the activity of AE381 would represent novel, potent immunomodulatory drugs which could be used to allosterically block autoantigenic peptide presentation on disease-linked MHC class II alleles.

5. References

[1] Bakke, O., Dobberstein, B., Cell **63,** 707 (1990) - [2] Roche, P., Cresswell, P., Nature **345,** 615 (1990) - [3] Blum, J. S., Cresswell, P., Proc. Natl. Acad. Sci. USA **85,** 3975 (1988) - [4] Roche, P., Cresswell, P., Proc. Natl. Acad. Sci. USA **88,** 3150 (1991) - [5] Nguyen, Q. N., Knapp, W., Humphreys, R. E., Human Immunol. **24,** 153 (1989) - [6] Riese, R. J., Wolf, P. R., Brömme, D. et al., Immunity **4,** 357 (1996) - [7] Daibata, M., Xu, M., Humphreys, R. E. et al., Mol. Immunol. **31,** 255 (1994) - [8] Xu, M., Capraro, G. A., Daibata, M. et al., Mol. Immunol. **31,** 723 (1994) - [9] Lu, S., Reyes, V. E., Lew, R. A. et al., Immunology **145,** 899 (1991) - [10] Adams, S., Humphreys, R. E., Eur. J. Immunol. **25,** 1693 (1995) - [11] Alsina, J., Rabanal, F., Giralt, E. et al., Tetrahedron Lett. **35,** 9633 (1994) - [12] Fields, G. B., Tian, Z., y Barany, G., in: Synthetic Peptides. A User's Guide, G. A. Grant (ed.), W. H. Freeman and Company, New York (1992) - [13] Stern, L. J., Brown, J. H., Jardetsky, J. T. et al., Nature **368,** 215 (1994) - [14] Ghosh, P., Ameya, M., Mellins, E. et al., Nature **378,** 457 (1995) - [15] Urban, R. G., Ghicz, R. M., Strominger, J. L., J. Exp. Med. **180,** 751 (1994) - [16] Kropshofer, H., Vogt, A. B., Stern, L. J. et al., Nature **270,** 1357 (1995)

Acknowledgement

Sharlene Adams was a postdoctoral fellow of the National Institute of Arthritis and Infectious Diseases (NIAID) while at University of Massachusetts Medical School.

Correspondence: Robert E. Humphreys, M.D., Ph.D., Antigen Express, Inc., One Innovation Drive, Worcester, MA 01605-4305 (USA)

Editors: Prof. Dr. Hans Georg Classen, Viktor Schramm. Editorial services: Waltraud Frey. Publisher: ECV · Editio Cantor Verlag für Medizin und Naturwissenschaften GmbH, P.O.B. 12 55, D-88322 Aulendorf (Germany), phone: (0 75 25) 9 40-0, fax: (0 75 25) 9 40-1 80; e-mail: redaktion@ecv.de; Internet: http://www.ecv.de. Printed by VEBU Druck GmbH, Am Reutele 18, D-88427 Bad Schussenried (Germany).

Delivery conditions: The journal is published monthly. Two volumes (I, II) per year. One volume includes 6 issues (supplementary issues free of charge). The journal is supplied by the publisher and by booksellers. Annual subscription rate (minimum 12 issues): 509.- DM plus postage; foreign annual subscription rate: 558.- DM plus postage. Sale per copy: 49.- DM plus postage. VAT always included. Free specimen copies are supplied by the publisher upon request.

Printed in Germany · ISSN 0004-4172