International Immunology, Vol 6, No. 5, pp. 751 - 759

Direct binding of autoimmune disease related T cell epitopes to purified Lewis rat MHC class II molecules

Irma Joosten[1,5], Marca H. M. Wauben[1], Moniek C. Holewijn[1], Konrad Reske[3], Lars Ø. Pedersen[2], Cyril F. P. Roosenboom[4], Evert J. Hensen[1], Willem van Eden[1] and Søren Buus[2]

[1]Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, Yalelaan 1, 3504 CL, Utrecht, The Netherlands
[2]Institute for Medical Microbiology and Immunology, Blegdamsvej 3B, DK-2200 Copenhagen, Denmark
[3]Institute for Immunology, Johannes Gutenberg University, Obere Zahlbacher Strasse 67, D-6500 Mainz, Germany
[4]National Institute of Public Health and Environmental Protection, PO Box 1, 3720 BA Bilthoven, The Netherlands
[5]Present address: Transplantation Serology Laboratory, University Hospital Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands

Key words: adjuvant arthritis, autoimmunity, class II affinity, competitor peptide, experimental autoimmune encephalitis, experimental autoimmune myasthenia gravis, experimental autoimmune uveoretinitis, rat MHC, peptide binding

Abstract

New strategies applied in the treatment of experimental autoimmune disease models involve blocking or modulation of MHC – peptide – TCR interactions either at the level of peptide – MHC interaction or, alternatively, at the level of T cell recognition. In order to identify useful competitor peptides one must be able to assess peptide – MHC interactions. Several well described autoimmune disease models exist in the Lewis rat and thus this particular rat strain provides a good model system to study the effect of competitor peptides. So far no information has been available on the peptide binding characteristics of the Lewis rat MHC class II RT1.B^{l} molecule. We have now developed a biochemical binding assay which enables competition studies in which the relative MHC binding affinity of a set of non-labelled peptides can be assessed while employing detection of biotinylated marker peptides by chemiluminescence. The assay is sensitive and specific. We have used this assay to determine the binding characteristics of several disease associated T cell determinants and their sequence analogues in the Lewis rat. Notably, most of the autoimmune disease associated peptide sequences tested were found to be intermediate to poor binders. Single amino acid substitutions at defined positions were sufficient to turn certain peptides into good binders. These results are relevant to the design of competitor peptides in the treatment of experimental autoimmune diseases.

Introduction

A novel approach in the treatment of CD4$^+$ T cell mediated experimental autoimmune diseases involves the inhibition or modulation of T cell responses by interfering with the formation of the trimolecular complex formed by MHC class II molecules, processed antigen (peptide) and TCR, by employing so-called competitor peptides. The formation of this trimolecular complex is an essential part of the final antigen-specific event in CD4$^+$ T cell activation (1 – 3) which can be manipulated in several ways. Competition at the level of peptide – MHC interaction has been demonstrated both *in vitro* and *in vivo* using MHC binding peptides as competitors (4 – 8). This type of competition interferes with all T cell responses restricted to the particular MHC molecule. *In vitro* competition at the level of the TCR has recently been suggested using peptides, which do not only bind to the relevant MHC molecule, but also bear close structural relationship with the stimulating peptide (9). This type of interference is expected

Correspondence to I. Joosten
Transmitting editor E Sercarz

Received 24 May 1993, *accepted* 14 February 1994

to have a far more limited, albeit more specific, effect. In any event, to work a competitor peptide needs to engage the MHC molecule in question.

The role of autoaggressive $CD4^+$ T cells in several experimental autoimmune disease models is evident, since T cell clones both in mice and in rats have been isolated which can transfer the disease into naive recipient animals (10,11,12). In the peptide induced experimental autoimmune encephalomyelitis (EAE) model in rodents, the use of competitor peptides already has proven successful in prevention of the disease (13–16). The Lewis rat is an inbred rat strain particularly prone to autoimmune diseases such as adjuvant arthritis (AA) (17), EAE (18), experimental autoimmune uveoretinitis (EAU) (19) and experimental autoimmune myasthenia gravis (EAMG) (20). Most disease associated $CD4^+$ T cell responses have so far been found to be restricted to a single MHC class II molecule, RT1.B^l (20–25). The Lewis rat is therefore a good model system to study the ffect of competitor peptides. However, no biochemical assay exists to determine the interaction between peptide and RT1.B^l, or any other rat MHC class II molecule. It has therefore not been possible to ascertain the affinity of RT1.B^l for any autoimmune disease related peptides or for any other peptide.

Consequently, we have developed a non-radioactive MHC class II—peptide binding assay which will allow us to rapidly screen peptides for their capacity to bind to the RT1.B^l molecule. To establish the assay we made use of an already well defined MHC–peptide combination [the binding of peptides hen egg lysozyme (HEL) 107–116 and dynorphin (DYN) 1–13 to purifed E^d molecules (26)], and we then used the new assay to describe a new MHC specificity, i.e. RT1.B^l. To our knowledge the direct binding of immunogenic peptides to RT1.B^l is the first reported in the rat. It enabled us to define the relative RT1.B^l binding capacity of autoimmune disease related T cell epitopes and their analogues. These results are of relevance to the design of MHC class II binding competitor peptides.

Methods

Peptides

Peptides used in the study were:

For the Lewis rat RT1.B^l:

MBP 72–85 (QKSQRSQDENPV)	Single amino acid lettering of an encephalitogenic T cell epitope from guinea pig myelin basic protein (MBP) associated with the EAE model and recognized by T cell line Z1a (21); the amino acids are numbered with respect to the bovine MBP that contains a glycine–histidine insertion at positions 76 and 77.
MBP 72–85S_{79}→A	Substitution analogues of MBP 72–85 (16).
MBP 72–85D_{81}→A	
MBP 72–85E_{82}→A	
MBP 72–85V_{85}→A	
MBP 72–85S_{79}→T	Encephalitogenic peptide from the rat MBP (22).
MBP 53–67 (RGSGKDSHHAARTTH)	EAE associated, secondary rat MBP epitopes (22).
MBP 87–99 (VHFFKNIVTPRTP), (D-locus binder)	
hsp65 180–188 (TFGLQLELT), hsp65 178–186 (SNTFGLQLE)	Stimulatory sequences from the 65 kDa mycobacterial heat shock protein recognized by arthritogenic T cell clone A2b associated with the AA model (27,28).
hsp65 180–188L_{183}→A	Substitution analogues of
hsp65 180–188L_{185}→A	hsp65 180–188 (16).
hsp65 178–186L_{183}→A	Substitution analogue of hsp65 178–186.
IRBP 579–591 (GECWLGGGVVPDA)	A bovine interphotoreceptor retinoid binding protein (IRBP) derived peptide, that can not induce EAU by itself, but can induce uveitogenic activity in IRBP sensitized T cells (24).
IRBP 1181–1191 (SWEGVGVVPDV),	Immunogenic and uveitogenic peptide from the IRBP associated with the EAU model (25).
AChR 101–116 (AIVHMTKLLLDYTGKI)	Immunodominant T cell epitope from the α subunit sequence of *Torpedo californica* electric organ acetylcholine receptor (AChR) (20).
OVA 323–339 (ISQAVHAAHAEINEAGR)	Stimulatory amino acid sequence of the OVA specific, RT1.B^l restricted T cell clone 1/C11.P7.

For the mouse I-E^d:

IBV 67–83 (QHGYWRRQARFKPGKGG)	T cell epitope from the nucleocapsid protein of infectious bronchitis virus (29).
HEL 107–116 (AWVAWRNRCK)	T cell epitope from HEL for binding to E^d (26).
DYN 1–13 (YGGFLRRIRPKLK)	Defined E^d binding sequence of DYN (26).

Peptides hsp65 180–188, hsp65 180–188L_{183}→A, MBP 72–85, MBP 72–85D_{81}→, IBV 67–83 and OVA 323–339Y were synthesized by standard solid phase Fmoc chemistry (30). Peptide IRBP 1181–1191 was synthesized using t-BOC

chemistry on an Applied Biosystems (Forster City, CA) Model 430A peptide synthesizer. The remaining peptides were synthesized by automated stimultaneous multiple peptide synthesis (31). The peptides were purified by HPLC and subsequently checked by fast atom bombardment mass spectrometry.

T cell lines and clones

T cell clone 1/C11.P7 with specificity for OVA 323 – 339 belongs to a series of RT1.B^l restricted OVA-specific T cell clones (B. Stier *et al.*, unpublished data). Briefly, Lewis rats were immunized with a total of 40 μg of OVA emulsified in physiologic saline and complete Freund's adjuvant, injecting 100 μl of emulsion per hind footpad. Ten days later cells from the draining lymph nodes were passed through nylon wool and were cultured at 2×10^6 cells/ml in Iscove's modified Dulbecco's medium supplemented with 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 IU penicillin, 100 μg/ml streptomycin and 5% FCS (complete medium) in the presence of 100 μg/ml OVA. Starting day 3 cells were subcultured in complete medium containing 2.5% 24 h culture supernatant Lewis rat spleen cells (5×10^6/ml) stimulated with concanavalin A (10 μg/ml; Sigma, St Louis, USA) (Con A SN). Cells were cloned by limiting dilution in the presence of 5×10^5 per well irradiated thymocytes (200 rad), 50 μg/ml OVA and 2.5% Con A SN. Serial re-stimulations were performed every 30 days using 2×10^5 T cells per well and 3×10^6 irradiated thymocytes per well in the presence of 50 μg/ml OVA, alternating with subculture in the presence of 2.5% Con A SN.

The isolation, maintenance and properties of the encephalitogenic T cell line Z1a, reactive with the 72 – 85 sequence of MBP, has been previously described (32). The isolation, maintenance and properties of the A2b helper T cell clone, reactive with the 180 – 188 amino acid sequence of mycobacterial hsp65, has been previously described (10). Briefly, the rat T cell lines were cyclically re-stimulated *in vitro* for 3 – 4 days with irradiated (3000 rad) thymocytes as antigen presenting cells (APC) and 10 μg/ml MBP for line Z1a or 10 μg/ml heat-killed *M. tuberculosis* for clone A2b and propagated for 6 or 7 days in Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY), supplemented with 10% FCS, 10% EL-4 supernatant (as IL-2 source), 2 mM L-glutamine, 2-mercaptoethanol, antibiotics and 1% non-essential amino acids. The generation, maintenance and characteristics of the IBV specific mouse T cell hybridoma MJB 100 is described elsewhere (33). They were cultured in Iscove' modified Dulbecco's medium (Gibco), supplemented with 10% FCS, 2 mM L-glutamine, 2-mercaptoethanol and antibiotics.

Cell culture for the production of MHC

For bulk culture, the rat T cell line Z1a was re-stimulated with 2.5 μg/ml Con A in the above described culture medium supplemented with 5% FCS for 3 – 4 days and propagated in culture medium supplemented with 5% FCS and 10% EL4 supernatant for 6 – 7 days. The H-2^d mouse B cell lymphoma cells, A20, were cultured in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine and 10% heat inactivated FCS.

Affinity purification of MHC molecules

Rat RT1.B^l was purified from cell lysates of Z1a T cell line. This line expresses MHC class II products: *de novo* synthesis has been established by metabolic cell labelling and subsequent immunoprecipitation (M. H. M. Wauben, unpublished data). Mouse I-E^d was purified from cell lysates of A20 B cell lymphoma cells. Cells were lysed at 10^8 cells/ml in PBS containing 1% NP-40, 25 mM iodoacetamide, 5 mM sodium orthovanadate and 0.5 mM phenylmethylsulfonyl fluoride. The lysates were cleared by centrifugation at 10,000 *g* for 20 min and subsequent passage over a 45 μm filter (Millipore, Bedford, USA). RT-1 B^l and I-E^d molecules were purified using the mAb OX6 and 14-4-4S respectively, coupled to CNBr-activated Sepharose-4B (Pharmacia-LKB, Uppsala, Sweden). The cell lysates were passed over the columns, which were washed with 20 column volumes of PBS containing 0.1% SDS, 0.5% NP-40, three column volumes PBS containing 0.05% NP-40 and finally three column volumes of PBS containing 1% *n*-octyl glucoside (OG). The MHC molecules were eluted with 0.05 M diethylamine in 0.15 M NaCl containing 1% OG (pH 11). The eluate was immediately neutralized with 50 μl/ml 2 M Tris – HCl, pH 6.3, and concentrated by vacuum dialysis. MHC preparations were kept in PBS, 1% OG and 0.1% azide.

Biotinylation of marker peptides

Peptides MBP 72 – 85 and IBV 67 – 83 were biotinylated with NHS-LC-Biotin (Pierce, Rockford, USA). Biotin was dissolved in double distilled water (1 mg/100 μl) and added in 2-fold molar excess to peptide dissolved in 0.5 M $NaHCO_3$, pH 9.0. After 2 h on ice, 2 M Tris – HCl buffer, pH 6.3, was added at 10% of the total volume to prevent unwanted reactivity of the remaining biotin. The protocol resulted in ~ 70% biotinylation of the peptide as estimated by ninhydrin reaction.

Peptides were biotinylated at higher ratio's in DMF. Thus we achieved higher labelling efficiency (up to 95%), but this did not result in improved signals upon testing in the binding assay.

MHC class II – peptide binding assay

Purified class II molecules (2 – 3 μM) were incubated with 5 – 500 nM of biotinylated peptides at pH 5 in a total volume of 15 μl for 48 h at room temperature in the presence of a protease inhibitor cocktail (final concentration 1 mM phenylmethylsulfonyl fluoride, 135 μM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 142 μM L-*p*-tosylamino-2-phenylethyl chloromethyl ketone, 1 mM *N*-ethyl maleimide, 8 mM EDTA, 1.3 mM 1,10-phenanthroline and 73 μM pepstatin A) and a final detergent concentration of 0.05% NP-40. The amount of MHC used appears appropriate since it is in the expected range known from mouse and human MHC studies using the radiolabelled peptide based assays (34,35). Since we have not actually defined the K_d values of the different rat MHC – peptide combinations, nor the active part of the purified MHC, this conclusion remains a tentative one. However, we have tested a dose range of MHC (1 – 4 μM) and found that in the competition assay optimal results were achieved with 3 μM of MHC. All binding studies were routinely performed at pH 5. For a largely qualitative assay such as ours, this pH was considered most appropriate (34 – 36). For competition studies concentrations of up to 100 nM of biotinylated peptide were used in combination with 0 – 100 μM of non-labelled competitor peptide.

The MHC – peptide mixtures were analyzed by SDS – PAGE under non-reducing conditions with the Laemmli buffer modified to a final SDS concentration of 0.1%, a slightly modified version

from that employed by Jardetzky *et al.* (37). The proteins were blotted onto nitrocellulose (Hybond-ECL; Amersham, Amersham, UK) using a semi-dry blotting system. After 1 h blocking with 5% dried milk in PBS – 0.05% Tween 20, the blot was incubated for 45 min with biotinylated streptavidin – horseradish peroxidase complexes in a 1:1000 dilution (Amersham). Detection of the biotinylated peptides was done by enhanced chemiluminescence using the Western blot ECL kit (Amersham). Blots were exposed for 1 h on preflashed Hyperfilm – ECL (Amersham).

An image processing technique was developed to quantify the results of the electrophoresis/blotting experiments (manuscript in preparation). Briefly, the autoradiograph was recorded with a videocamera (MO High Resolution CCD; High Technology Holland BV, Eindhoven, The Netherlands) and digitized by a frame-grabber (PC Vision Plus; Image Technology USA., Worburn, MA). The digitized picture was processed and analyzed using the computer program TIM (version 3.20; Difa Measuring Systems, TEA, Breda, The Netherlands). The spots on the autoradiograph were quantified by multiplying the density of the pixels constituting the spots with the surface of the spots. Results were expressed as spot intensity [= pixel^2 × (standard maximum grey value – mean grey value of the spot)]. Inhibition curves were generated from these results and the concentration of competitor peptide at which 50% inhibition was achieved (IC_{50} value) was extrapolated from these curves. Subsequently, these figures were converted into plusses to reflect the largely qualitative nature of the assay and the peptides were ranked with regard to their relative binding affinity for the RT1.B^l molecule.

Functional competition assay

Competition assays were done both with fixed and non-fixed APC. For fixed APC rat thymocytes or mouse A20 B lymphoma cells were fixed in 0.5% gluteraldehyde and subsequently pulsed for 3 h at pH 5 with a dose range of competitor peptide and a fixed, suboptimal concentration of stimulatory peptide (OVA 323 – 339Y, MBP 72 – 85 or IBV 67 – 83) in the presence of a protease inhibitor mix (composition and concentration as above). After extensive washing, antigen specific T cells (1/C11.P7 or Z1a) or hybridoma cells (MJB100) were added. Each well contained 1×10^6 thymocytes or 1×10^5 A20 cells together with 2×10^4 rat T cells or mouse hybridoma cells respectively. After 3 days of culturing the rat cells were pulsed for 18 h with [^{3}H]thymidine and subsequently harvested. Proliferation of the T cell lines was measured by [^{3}H]thymidine incorporation followed liquid scintillation (Beckman). IL-2 production by the I-E^d restricted MJB100 hybridoma cells was measured by harvesting the 24 h culture supernatant, to which 5×10^3 CTLL cells were added as IL-2 responders. After a further 24 h of culture, [^{3}H]thymidine was added and the incorporated radioactivity was measured 18 h later. Additionally for the rat, system competition studies were performed using non-fixed APC. A dose range of competitor peptides was added to cultures containing 2×10^4 T cells (1/C11.P7, Z1a or A2b) and 1×10^6 irradiated rat thymocytes 2 h before the addition of a constant, suboptimal amount of stimulatory peptide (OVA 323 – 339Y, MBP 72 – 85 or hsp65 180 – 188). Proliferation was measured by [^{3}H]thymidine incorporation.

Results

Detecting peptide – MHC class II interaction by chemiluminescence

As ligands for each of the two MHC molecules used to establish the assay, RT1.B^l and I-E^d, we selected previously defined autoimmune disease or viral disease related T cell epitopes, i.e. MBP 72 – 85 and IBV 67 – 83 respectively. These peptides were biotinylated with a 2-fold molar excess of water soluble biotin. Binding of biotinylated MBP 72 – 85 to RT1.B^l and biotinylated IBV 67 – 83 to I-E^d was demonstrated as shown in Fig. 1. The detection method was highly sensitive since concentrations of only 3 μM MHC incubated with as little as 5 nM (IBV 67 – 83) or 50 nM (MBP 72 – 85) of biotinylated peptide yielded a signal. The binding was allele specific since incubation of 500 nM (10 – 100 times excess) of either of the two peptides with the irrelevant MHC molecule did not result in binding (lanes 7). Omitting the biotinylated peptide or the MHC did not yield any

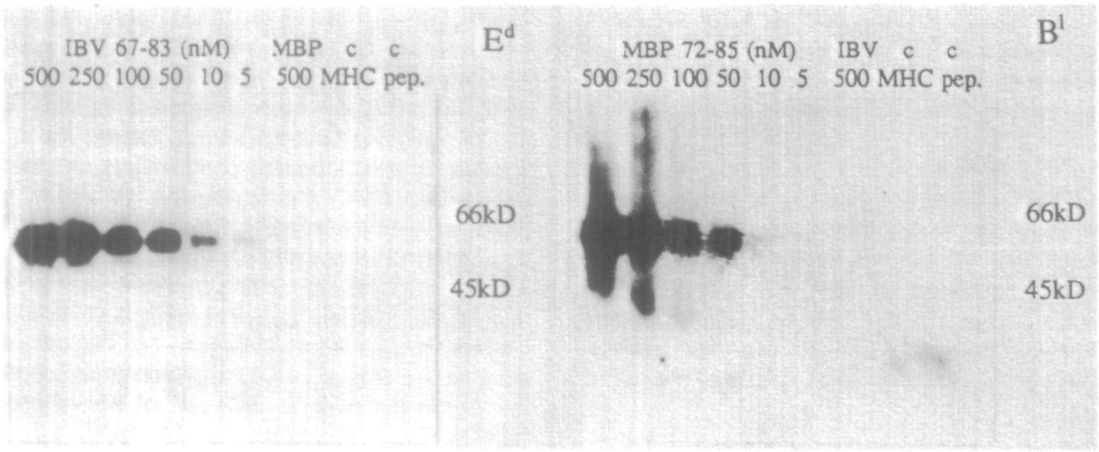

Fig. 1. Direct binding of a dose range (nM) biotinylated IBV 67 – 83 to 3 μM E^d (left) and biotinylated MBP 72 – 85 to 3 μM RT1.B^l (right). Allele-specific binding was demonstrated in lanes 7: incubation of 500 nM of either of the two peptides with the reverse MHC type did not result in binding. The controls (c) are MHC without the addition of peptide and peptide without the addition of MHC.

background signal (lanes c). The allele specificity of peptide binding could potentially be changed by the biotinylation. To exclude this possibility for each of the two peptide – MHC combinations in questions, we used a dose range of non-labelled MBP 72 – 85 or IBV 67 – 83 to inhibit the binding of either biotinylated IBV 67 – 83 to I-E^d or biotinylated MBP 72 – 85 to RT1.B^l respectively. In both cases the signal was not inhibited, thus confirming that the non-biotinylated peptides did not bind to the non-relevant MHC type either (data not shown).

The possible effect of biotinylation on the binding affinity of peptides for MHC is still of concern. Once a biotinylated peptide has been shown to bind to the MHC class II molecule in question in an allele specific manner then the assay can be converted into a competition assay. Such a competition assay measuring the ability of non-labelled peptides to block the binding of the biotinylated peptide to the MHC class II molecule will be independent of the effect of biotinylation The low concentration of marker peptides required to obtain a clear chemiluminescence signal enabled us to assess the binding capacity of non-labelled peptides by competition. We performed competition studies with the known strong I-E^d binders DYN 1 – 13 and HEL 107 – 116 as non-biotinylated competitor peptides. DYN 1 – 13 is known to bind with higher affinity to I-E^d than HEL 107 – 116 (26). In agreement herewith we here show that the non-labelled DYN 1 – 13 inhibited the binding of biotinylated IBV 67 – 83 binding to I-E^d at a lower concentration than non-labelled HEL 107 – 116 did (see Fig. 2). To further quantify the results a digitized picture of the autoradiographs was made. A resolution was achieved of 512 × 512 pixels and a maximum grey value number of 256. This extends beyond the resolution of the human vision. For each of the competitor peptides, a dose – response curve was made and from this the concentration at which 50% inhibition was reached was extrapolated. For DYN 1 – 13 this dose was 2 μM, whereas for HEL 107 – 116 the dose was ~5 μM (Fig. 3) The inhibition curves were reproducible from gel to gel

Comparison of the biochemical competition assay with a functional competition assay

The biochemical assay was further confirmed by comparing it with a functional inhibition study in which fixed A20 cells were incubated with IBV 67 – 83 and competitor peptide at pH 5 in the presence of a protease inhibitor cocktail. The responses of the IBV 67 – 83 specific, I-E^d restricted T hybridoma cells were used to measure the inhibitory activity of the two competitor peptides DYN 1 – 13 and HEL 107 – 166 (Fig. 4). DYN 1 – 13 blocked the response by 50% at ~70 μM, whereas HEL 107 – 116 blocked the response by 50% at ~175 μM, thus showing that the biochemical binding assay yields similar results as the T cell functional competition assay

Peptide binding to the rat RT1 B^l

We now assessed the relative MHC class II binding affinity of peptides relevant for the four autoimmune disease models in the Lewis rat, their analogues and a single non-disease related peptide (OVA 323 – 339). T cell responses have been identified as being RT1.B^l restricted for all the peptides tested (20 – 25), with the exception of the response to the MBP 87 – 99 which is D-locus restricted (22). A dose range of non-labelled peptides was incubated together with 100 nM of biotinylated MBP 72 – 85 and 3 μM of affinity purified RT1.B^l at pH 5, and the resulting binding determined as described above. The degree of inhibition was further quantified by image processing. From the dose – response curves obtained for each of the peptides the concentration at which 50% inhibition was achieved was extrapolated. The peptides could be ranked into five broadly defined categories. Good binders (+ + +, with IC_{50} values of 1 – 10 μM) were the OVA 323 – 339 peptide, and single alanine substitution analogues of the MBP 72 – 85, hsp 178 – 186 and hsp 180 – 188 peptides; intermediate binders (+ +, with IC_{50} values of 10 – 100 μM) were the natural MBP 72 – 85 immuno-

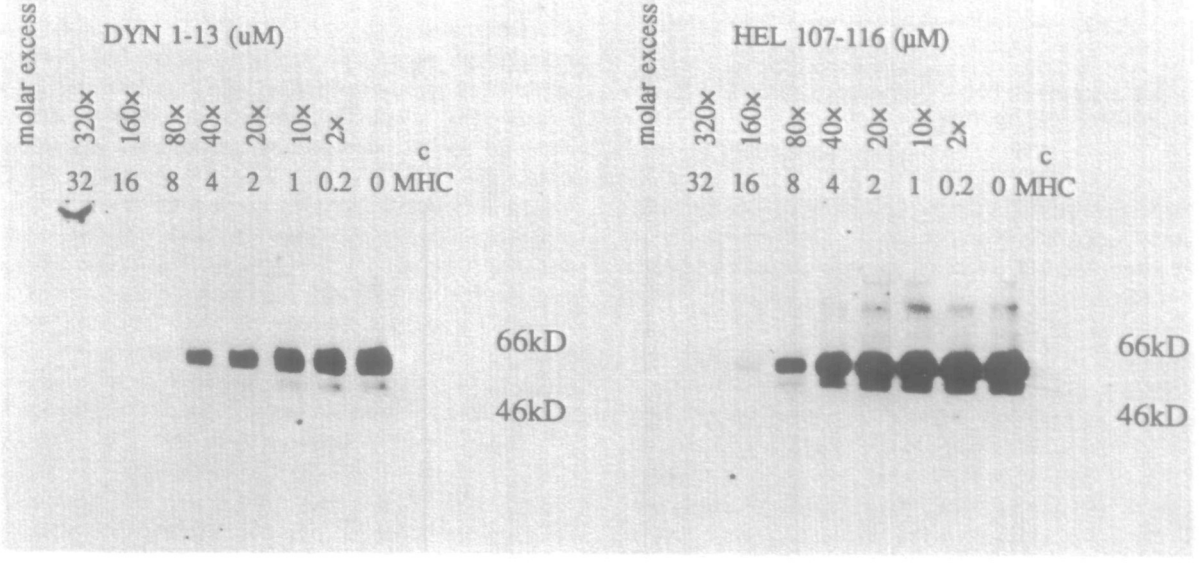

Fig. 2. Inhibition of the binding of 100 nM biotinylated IBV 67 – 83 to 3 μM purified I-E^d by a dose range of non-labelled DYN 1 – 13 or HEL 107 – 116. The control (c) is MHC without the addition of peptide.

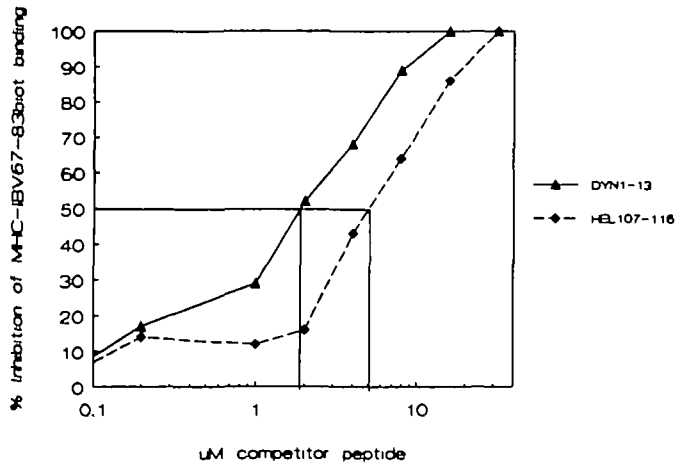

Fig. 3. Inhibition of the binding of 100 nM biotinylated IBV 67 – 83 to 3 μM purified I-E^d by a dose range of non-labelled DYN 1 – 13 (▲) or HEL 107 – 116 (♦) quantitated by image processing. For both peptides the 50% inhibitory dose is indicated.

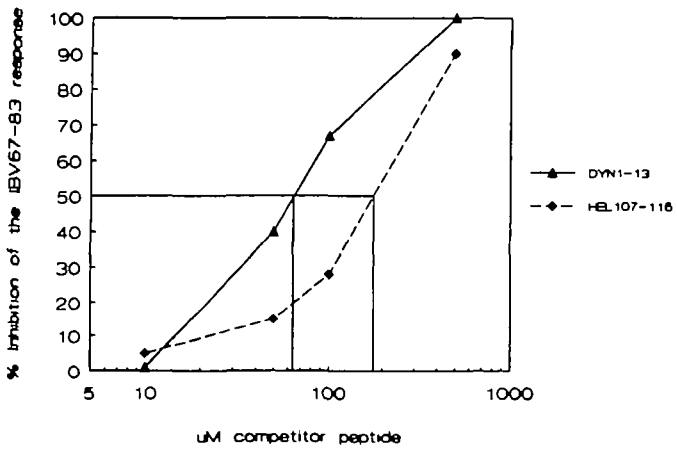

Fig. 4. Inhibition of T cell hybridoma (MJB100) IL-2 production in the mouse system. Fixed A20 cells were pulsed with 5 μM stimulatory peptide (IBV 67 – 83) and a dose range of competitor peptide [DYN 1 – 13 (▲) and HEL 107 – 116 (♦)] at pH 5 in the presence of a protease inhibitor cocktail. For both peptides the 50% inhibitory dose is indicated. Results exemplify at least two tests per peptide.

dominant epitopes from guinea pig and rat, a guinea pig MBP 72 – 85 alanine substitution analogue (V_{82}→A), and the natural hsp65 178 – 186 and 180 – 188 sequences recognized by the athritogenic T cell clone A2b. Notably, among the poor binders (+, with IC_{50} values of 100 – 300 μM) were the IRBP 579 – 591 peptide, inducing uveitogenic activity in IRBP sensitized T cells, and the guinea pig MPB 53 – 67 peptide, comprising a secondary EAE associated epitope; the uveitogenic IRBP 1181 – 1191 peptide and the immunodominant EAMG associated AChR 101 – 116 peptide were found to be very poor binders (+/–), even at 256 μM not more than a slight inhibition was observed. Also, two alanine substitution analogues, hsp65 180 – 188L_{185}→A and MBP 72 – 85E_{82}→A, were found to be poor binders. As expected, no inhibition was observed for the secondary rat MBP 87 – 99 epitope from which it is known that

Table 1. Competition for binding to purified RT1.B^l measured in a direct binding assay

Peptide	Relative binding affinity[a]
OVA 323 – 339	+ + +
MBP 72 – 85S_{79}→A	+ + +
hsp65 178 – 186L_{183}→A	+ + +
MBP 72 – 85D_{81}→A	+ + +
hsp65 180 – 188L_{183}→A	+ + +
MBP 72 – 85	+ +
MBP 72 – 85S_{79}→T (rat)	+ +
MBP 72 – 85V_{85}→A	+ +
hsp65 178 – 186	+ +
hsp65 180 – 188	+ +
IRBP 579 – 591	+
hsp65 180 – 188L_{185}→A	+
MBP 53 – 67	+
MBP 72 – 85E_{82}→A	+/–
IRBP 1181 – 1191	+/–
AChR 101 – 116	+/–
MBP 87 – 99 (D-locus binder)	–

[a]Inhibition of the binding of 100 nM biotinylated MBP 72 – 85 to 3 μM of affinity purified RT1.B^l. Different dose ranges of 0 – 300 μM of competitor peptide were used. The IC_{50} values were extrapolated from the inhibition curves each comprising six to eight data points. Quantitation was by image processing of the autoradiographs. The results represent two to four experiments for each peptide Reproducibility was high. IC_{50} values from 0 to 10 μM are indicated by + + +; IC_{50} values from 10 to 100 μM are indicated by + +; IC_{50} values from 100 to 300 μM are indicated by +; peptides that showed inhibition, but had IC_{50} values clearly >300 μM are indicated by +/–; MBP 87 – 99 showed no inhibition at all, indicated by –. The original autoimmune disease related sequences are underlined.

the response of encephalitogenic T cells against this epitope is D-locus restricted.

A selected subset of the peptides was tested in functional competition assays to confirm the immunological relevancy of the direct binding data and to compare this assay to the functional competition assay. The functional assay was performed with either fixed or non-fixed APC, the former at pH 5 and in the presence of a protease inhibitor mix. Results are shown in Table 2. The concentration of competitor peptide needed to obtain 50% inhibition (IC_{50}) was compared with the IC_{50} of an arbitrarily chosen standard competitor peptide. This relative inhibitory concentration can be used to make comparisons between different T cell read-outs. The non-analogous OVA-specific, RT1.B^l restricted T cell read-out was used to assess the *in vitro* competitor function of the two strongest binders MBP 72 – 85D_{81}→A and hsp65 180 – 188L_{183}→A. Using either of the two functional competition assays (fixed or non-fixed APC) the results confirmed the earlier biochemical findings that MBP 72 – 85D_{81}→A is a better RT1.B^l binder than hsp65 180 – 188L_{183}→A. Similar relative IC_{50} for the MBP 72 – 85D_{81}→A and hsp65 180 – 188L_{183}→A binding to RT1.B^l binding were obtained using the MBP 72 – 85 specific, RT1.B^l restricted Z1a T cells for the functional read-out. In contrast, using the hsp 180 – 188 specific, RT1.B^l restricted T cell clone, A2b, it was shown that within the given experimental design the IC_{50} value

Table 2. Inhibition of T cell proliferation by competition for antigen presentation

T cells	APCs	Stimulatory peptide	Competitor peptide	IC_{50}[a] (μM)	Relative IC_{50}[b]
1/C11.P7	fixed	OVA 323–339Y (2.8 μM)	MBP 72–85D_{81}→A	166.5	1
			hsp65 180–188L_{183}→A	236.5	1.4
	non-fixed	OVA 323–339Y (28 nM)	MBP 72–85D_{81}→A	17 8	1
			hsp65 180–188L_{183}→A	20.9	1.2
Z1a	fixed	MBP 72–85 (17.5 μM)	MBP 72–85D_{81}→A	253.1	1
			hsp65 180–188L_{183}→A	440.0	1.7
	non-fixed	MBP 72–85 (7 μM)	MBP 72–85D_{812}→A	39.4	1
			hsp65 180–188L_{183}→A	60.1	1.5
			hsp65 180–188	>200	>5.1
			IRBP 1181–1191	>200	>5.1
A2b	non-fixed	hsp65 180–188 (0.5 μM)	MBP 72–85D_{81}→A	51.1	1
			hsp65 180–188L_{183}→A	14 8	0.3
			MBP 72–85	>200	>3.9
			IRBP 1181–1191	>200	>3.9

The data are from a representative experiment.
[a]IC_{50} is the concentration of competitor peptide (μM) resulting in 50% inhibition of the proliferative response under the given experimental conditions
[b]Relative IC_{50} is the IC_{50} of a given competitor peptide divided by the IC_{50} of peptide MBP 72–85D_{81}→A.

of the competitor analogue hsp65 180–188L_{183}→A was much lower than that of the MBP 72–85D_{81}→A peptide, indicating a more efficient inhibition by hsp65 180–188L_{183}→A. Since both the direct binding assay and the functional assay in the non-analogous systems indicate that MBP 72–85D_{81}→A is the better MHC binder, the observed strong functional hsp65 180–188L_{183}→A inhibition of the A2b response appears to represent more than merely MHC blockade. The functional inhibition assays also demonstrated that MBP 72–85, hsp65 180–188 and IRBP 1181–1191 at the given concentrations are poor competitors. Thus, in the non-analogous read-outs the same ranking of the peptides is obtained with the functional competition assays as with the biochemical competition assay.

Discussion

The modulation of autoimmune disease by interfering with the $CD4^+$ T help response through MHC blockade is a novel approach. It has recently been observed that *in vivo* T cell activation can be prevented by co-immunization with stimulator peptide and MHC binding competitor peptides (8). Successful attempts to interfere with the onset or occurrence of autoimmune diseases by immunization with non-pathogenic competitor peptides have been achieved in the rodent EAE model (13–16). The Lewis rat presents an ideal model species for studying autoimmune diseases since a number of experimental autoimmune diseases can be induced in this rat strain. Disease associated T cell determinants in the Lewis rat have been defined for most models (17–22,26) and, with a single exception, the different autoimmune T cell responses are restricted to the same MHC class II specificity, RT1.B^l. The same competitor peptide specific for RT1.B^l can therefore be studied in several different autoimmune diseases models in the same experimental animal strain. However, to identify such RT1.B^l binding competitor peptides and to understand the possible mechanism of action it becomes essential to measure directly the binding of competitor peptides to MHC class II molecules as well as the binding of peptides involved in activating autoreactive T cell responses.

Our currently used method employs the detection of biotinylated peptides by chemiluminescence, whereby we have achieved stable and non-reactive labelling of peptides, yet maintained the required sensitivity. The assay has been established for both the mouse I-E^d and the rat RT1.B^l. The results obtained show sensitive and allele-specific binding. The method enables competition studies in which the relative binding affinities of a set of non-labelled peptides can be easily assessed. The assay is thus especially useful for the screening of sets of peptides, looking for good binders, in its present form it is less well suited for kinetic studies. A particular asset of the analysis is that it solves the problem of high non-specific backgrounds observed for some peptides in the gel filtration analysis, due to aggregation of peptide molecules. When starting with a not yet tested MHC specificity this can severely hamper the search for good binders. The high separation power of the SDS–PAGE analysis circumvents this problem. Furthermore, the sensitivity of our test system is high allowing the use of very low concentrations of labelled marker peptide, comparable to what is used in peptide binding assays based on radiolabelled peptides (34,35). This is in contrast to the high concentrations of labelled peptide necessary to detect binding of biotin labelled peptide to whole cells and detected by FACS analysis (38,39 and M. H. M. Wauben *et al.*, unpublished results). The very high concentration of labelled peptides needed to perform the direct binding assay based on FACS analysis makes it difficult to convert the assay into the versatile competition assay, something which can reliably be achieved with the present assay. Recently, potentially very useful biotin based assays have been developed (40–42). It should be noted, however, that the successful outcome of these latter assays is in part dependent on the particular combination of MHC specificity and anti-class II mAb. To be more specific, the conformational binding properties of the mAb and MHC molecules can interfere in the measurements of MHC–peptide complex formation. In a species like the rat where a very limited choice of mAb is available, this could seriously hinder the development of a binding assay. In our present study this is of no concern.

The results obtained with the biochemical competition assay were confirmed by functional competition assay for both the mouse and the rat systems. It is obvious that the ability to inhibit MHC binding and subsequently inhibit specific T cell stimulation varies greatly from peptide to peptide. The direct binding assay will yield information which is important in the generation of a MHC blocking strategy.

With respect to the relative binding affinity of the autoimmune associated determinants it is of interest to note that, firstly, none of the natural sequences from autoimmune disease associated proteins were among the highest binders (1 – 10 μM range). Secondly, from the peptides with direct disease inducing potential, the guinea pig MBP 72 – 85 peptide and the IRBP 1181 – 1191 peptide, the MBP peptide was found to be a high 'intermediate' binder, as opposed to the IRBP peptide, which was a very poor binder. Also the immunodominant AChR epitope and the secundary IRBP and MBE epitopes were found to be poor binders. Notwithstanding their apparent poor binding affinity, these peptides have the potential to play crucial roles in the development of the disease (20,22,24). It was argued by Lamont *et al.* (43) and Wall *et al.* (44) that in the mouse EAE model MHC binding affinity is correlated with encephalitogenicity and that a high MHC binding affinity may be a necessary characteristic of an encephalitogenic determinant. In our study it appears that in the Lewis rat the major encephalitogenic determinants are intermediary binders, i.e. ~10-fold less effective than the highest binder, the non-autoimmune disease related OVA peptide. Furthermore, a substitution analog of MBP 72 – 85 with about tenfold increased MHC binding affinity (MBP72 – $85S_{79}$ – A) and capable of inducing disease did not show an increased encephalitogenic potential as compared to the natural sequence (M. H. M. Wauben *et al.*, submitted for publication), suggesting that at least in this model high MHC binding affinity is not directly correlated with disease inducing potential. The finding that the natural peptide sequences relevant for the AA, EAU and EAMG models were low-intermediates to poor binders, is more in line with the work of Gammon and Sercarz (45), in which it is hypothesized that pathogenic determinants on self antigens resemble so-called minor determinants and are poorly presented, resulting in the evasion of tolerance induction by T cells with specificity for these determinants. In view of the results from the mouse EAE model (43,44), it is of importance to establish to what degree this concept is dependent on the particular disease and/or self antigen involved. Consequently, we are currently in the process of extending our panel of autoimmune disease related and non-related peptides, to be tested in the direct MHC binding assay.

By generating single amino acid substitution analogues of disease associated T cell determinants we were able to define peptides with improved MHC binding affinity, to be used for blocking purposes. The now apparent relative intermediate to poor binding affinity of the disease associated peptides has supported and facilitated this approach. An alternative strategy using analogues involves engagement without stimulation of the TCR (9). An example of this may be the strong inhibitory *in vitro* (tested in an analogous system) and *in vivo* capacity of hsp65 180 – $188L_{183}$ – A (16), which cannot be based on MHC blockade alone since in the direct binding assay as well as in the non-analogous functional assay, MBP72 – $85D_{81}$ – A was shown to have a higher affinity for MHC. In conclusion, this rat MHC – peptide binding assay may prove very valuable in the search for competitors of the formation of the trimolecular T cell stimulatory complex.

Acknowledgements

The authors wish to thank P. van Kooten for excellent technical advice. Dr R. van der Zee, A. Noordzij and Dr G. van der Werken are acknowledged for peptide synthesis and analysis. Professor I. Cohen is acknowledged for the use of clones A2b and Z1a. Dr A. Kozhich is acknowledged for the gift of the IRBP peptide. We also thank Dr A. Boots for critically reading the manuscript. The research of I. J. has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. Supported by grants from the Netherlands Organization for Scientific Research (NWO) and the European Concerted Action on immunopathogenesis and immunotherapy of chronic arthritis. Also, supported by grants from the Danish MRC, the Carlsberg and Novo Foundations, and the Centre for Signal peptide research.

Abbreviations

AA	adjuvant arthritis
AChR	acetyl choline receptor
Con A	concanavalin A
DYN	dynorphin
EAE	*experimental autoimmune encephalomyelitis*
EAU	experimental autoimmune uveoretinitis
EAMG	experimental autoimmune myasthenia gravis
HEL	hen egg lysozyme
hsp65	65 kDa heat shock protein of *Mycobacterium bovis*
IBV	infectious bronchitis virus
IRBP	bovine interphotoreceptor retinoid binding protein
MBP	myelin basic protein
OG	*n*-octyl-glucoside
OVA	chicken egg ovalbumin
SN	supernatant

References

1 Ashwell, J. D. and Schwartz, R. N. 1987. T-cell recognition of antigen and the Ia molecule as a ternary complex. *Nature* 320:176.
2 Watts, T. H. and McConnell, H. M. 1986. High-affinity fluorescent peptide binding to I-A^d in lipid membranes. *Proc. Natl Acad. Sci. USA* 83:9660.
3 Allen, P. M., Babbitt, B. and Unanue, E. 1987. T cell recognition of lysosyme—the biochemical basis of presentation. *Immunol. Rev.* 98:172.
4 Babbitt, B. P., Allen, P. M., Matsueda, G., Habert, E. and Unanue, E. R. 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* 317:359.
5 Buus, S. and Werdelin, O. 1986. Oligopeptide antigens of the angiotensin lineage compete for presentation by paraformaldehyde treated accessory cells to T cells. *J. Immunol.* 136.459.
6 Adorini, L., Muller, S., Cardinaux, F., Lehman, P. V., Falcioni, F. and Nagy, Z. A. 1988. *In vivo* competition between self peptides and foreign antigens in T cell activation. *Nature* 334:623.
7 Muller, S., Adorini, L., Juretic, A. and Nagy, Z. A. 1990. Selective *in vivo* inhibition of T cell activation by class II MHC binding peptides administered in soluble form. *J. Immunol.* 145:4006.
8 Guery, J. C., Sette, A., Leighton, J., Dragomir, A. and Adorini, L. 1992. Selective immunosuppression by administration of major histocompatibility complex (MHC) class II binding peptides. I. Evidence for *in vivo* MHC blockade preventing T cell activation. *J. Exp. Med.* 175:1345.
9 DeMagistris, M. T., Alexander, J., Coggeshall, M., Altman, A., Gaeta, F. C. A., Grey, H. M. and Sette, A. 1992. Antigen analog-Major histocompatibility complexes act as antagonists at the T cell receptor. *Cell* 68:625.

10 Holoshitz, J., Naparstek, Y., Ben-Nun, A. and Cohen, I. R. 1983. Lines of T lymphocytes induce or vaccinate against autoimmune arthritis *Science* 291:56.

11 Holoshitz, J., Frenkel, A., Ben-Nun, A. and Cohen, I. R. 1983. Autoimmune encephalomyelitis (EAE) mediated and prevented by T lymphocyte lines directed against diverse antigen determinants of myelin basic protein. Vaccination is determinant specific. *J. Immunol.* 131:2810.

12 Zamvil, S., Nelson, P., Trotter, J., Mitchell, D., Knobler, R., Fritz, R. and Steinman, L. 1985 T cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination *Nature* 317.355.

13 Sakai, K., Zamvil, S. S., Mitchell, D. J., Hodgkinson, S., Rothbard, J. B. and Steinman, L. 1989. Prevention of experimental encephalomyelitis with peptides that block interaction of T cells with MHC proteins *Proc. Natl Acad. Sci. USA* 86:9470.

14 Wraith, D. C., Smilek, D. E., Mitchell, D. J., Steinman, L. and McDevitt, H. O. 1989 Antigen recognition in autoimmune encephalomyelitis and the potential for peptide mediated immunotherapy. *Cell* 59:247.

15 Smilek, D. E., Wraith, D. C., Hodgkinson, S., Steinman, L. and McDevitt, H. O. 1991. A single amino acid change in a myelin basic protein peptide confers the capacity to prevent rather than induce experimental autoimmune encephalomyelitis. *Proc. Natl Acad. Sci. USA* 88:9633

16 Wauben, M. H. M., Boog, C. J. P., van der Zee, R., Joosten, I., Schlief, A. and van Eden, W. 1992. Disease inhibition by major histocompatibility complex binding peptide analogues of disease-associated epitopes: more than blocking alone *J. Exp. Med.* 176–677

17 Pearson, C. M. 1956. Development of arthritis, periarthritis and periostitis in rats given adjuvant. *Proc. Soc. Exp Biol. Med.* 91:95.

18 Ben-Nun, A. and Cohen, I. R. 1982. Experimental autoimmune encephalomyelitis (EAE) mediated by T cell lines: process of selection of lines and characterization of the cells. *J. Immunol.* 129.303.

19 Grey, I., Mochizuki, M. and Nussenblatt, R. B. 1986. Retinal specific antigens and immunopathogenic processes they provoke. *Prog Retinal Res.* 5:75

20 Fuji, Y. and Lindstrom, J. 1987. Specificity of the T cell immune response to acetylcholine receptor in experimental autoimmune myasthenia gravis *J. Immunol.* 140 1830.

21 Chou, Y. K., Vandenbark, A. A., Jones, R., Hashim, G. and Offner, H. 1989. Selection of encephalitogenic rat T lymphocyte clones recognizing an immunodominant epitope on myelin basic protein *J. Neurosci Res.* 22:181.

22 Offner, H., Hashim, G. A., Celnik, B., Glang, A., Li, X., Burns, F. R., Shen, N., Heber-Katz, E. and Vandenbark, A. A. 1989. T cell determinants of myelin basic protein include a unique encephalitogenic I-E restricted epitope for Lewis rats. *J. Exp. Med.* 170:355.

23 Boots, A. M. H., Van Lierop, M. J., Wauben, M. H. M., Van Kooten, P. J. S., Hensen, E. J. and Van Eden, W. 1991. CD4 rat × rat and mouse × rat T cell hybridomas produced by fusion of established T cell lines and clones to W/Fu (C58NT)D. *J Immunol. Methods* 144:1

24 Kotake, S., Wiggert, B., Zhang, X., Redmond, T. M., Chader, G. J and Gery, I. 1990. Stimulation *in vitro* of lymphocytes for induction of uveoretinitis without any significant proliferation. *J. Immunol* 145:534.

25 Sanui, H., Redmond, T. M., Kotake, S., Wiggert, B., Hu, L., Margalit, H., Berzofsky, J. A., Chader, G. J and Gery, I. 1989. Identification of an immunodominant and highly immunopathogenic determinant in the retinal interphotoreceptor retinoid-binding protein (IRBP). *J. Exp. Med.* 169:1947.

26 Sette, A., Adorini, L., Apella, E., Colon, S. M., Miles, C., Tanaka, S., Ehrhardt, C., Doria, G., Nagy, Z. A., Buus, S and Grey, H. M. 1989. Structural requirements for the interaction between peptide antigens and I-E^d molecules. *J. Immunol.* 143:3289.

27 Van Eden, W., Thole, J. E. R., Van der Zee, R., Noordzij, A., Van Embden, J. D. A., Hensen, E. J. and Cohen, I. R. 1988. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature* 331:171.

28 Wauben, M. H. M., Van der Zee, R., Joosten, I., Boog, C. J. P., Van Dijk, A. M. C., Holewijn, M. C., Meloen, R. H. and Van Eden, W. 1993. A peptide variant of an arthritis related T cell epitope induces T cells that recognize this epitope as a synthetic peptide but not in its naturally processed form. *J. Immunol.* 12:5722.

29 Boots, A. M. H., Kusters, J. G., Van Noort, J. M., Zwaagstra, K. A., Rijke, E., van der Zeyst, B. A. M. and Hensen, E. J. 1991. Localization of a T cell epitope within the nucleocapsid protein of avian coronavirus. *Immunology* 74:8.

30 Steward, J. M. and Young, J. D. 1984. *Solid Phase Peptide Synthesis.* Pierce Chemical Co., Rockford, IL.

31 Van der Zee, R., Wauben, M. H. M., Lots, T. H. A. and Van Eden, W. 1992 Simultaneous multiple peptide synthesis (SMPS) for the analysis of T cell epitopes. *J. Cell. Biochem* 16D:83.

32 Ben-Nun, A., Wekerle, H. and Cohen, I. R. 1981. The rapid isolation of clonable antigen specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur. J. Immunol.* 11.195.

33 Boots, A. M. H., Van Lierop, M. J., Kusters, J. G., Van Kooten, P. J. S., Van der Zeyst, B. A. M. and Hensen, E. J. 1990. MHC class II restricted T-cell hybridomas recognizing the nucleocapsid protein of avian coronavirus. *Immunology* 72:10.

34 Sette, A., Southwood, S., O'Sullivan, D., Gaeta, F. C. A., Sidney, J. and Grey, H. M. 1992. Effect of pH on MHC class II–peptide interactions. *J. Immunol.* 148:844.

35 Mouritsen, S., Stryhn Hansen, A., Laub Petersen, B. and Buus, S. 1992. pH dependence of the interaction between immunogenic peptides and MHC class II molecules. Evidence for an acidic compartment being the organelle of interaction. *J. Immunol.* 148:1438.

36 Jensen, P. E. 1990. Regulation of antigen presentation by acidic pH. *J. Exp. Med.* 171:1779

37 Jardezky, T. S., Gorga, J. C., Busch, R., Rothbard, J., Strominger, J. L. and Wiley, D. C. 1990 Peptide binding to HLA-DR1: a peptide with most residues substituted to alanine retains MHC binding. *EMBO J.* 6:1797.

38 Busch, R., Strang, S., Howland, K. and Rothbard, J. B. 1990. Degenerate binding of immunogenic peptides to HLA-DR proteins on B cell surfaces. *Int. Immunol.* 2.443.

39 Mozes, E., Dayan, M., Zisman, E., Brocke, S., Licht, A. and Pecht, I. 1989. Direct binding of a myasthenia gravis related epitope to MHC class II molecules on living murine antigen presenting cells. *EMBO J.* 8.4049.

40 Jensen, P. 1991. Enhanced binding of peptide antigen to purified class II major histocompatibility glycoproteins at acidic pH *J. Exp. Med.* 174:1111

41 Reay, P. A., Wettstein, D. A. and Davis, M. M 1992. pH dependence and exchange of high and low responder peptides binding to a class II molecule. *EMBO J.* 8:2829.

42 Buelow, R., Paborsky, L. R., van Schooten, W. C. A., Kummerehl, T. J., Schreifels, R., Marshall, K., Mayer, J. and Rothbard, J. B. 1993 Comparison of three different forms of HLA-DR4Dw4 proteins. *Eur. J Immunol.* 23:69.

43 Lamont, A. G., Sette, A., Fujinami, R., Colon, S. M., Miles, C. and Grey, H. M. 1990. Inhibition of experimental autoimmune encephalomyelitis induction in SJL/J mice by using a peptide with high affinity for IA^s molecules. *J. Immunol.* 145:1687.

44 Wall, M., Southwood, S., Sidney, J., Oseroff, C., del Guericio, M., Lamont, A. G., Colon, S. M., Arrhenius, T., Gaeta, F. C. A. and Sette, A. 1992. High affinity for class II molecules as a necessary but not sufficient characteristic of encephalitogenic determinants. *Int. Immunol.* 4:773.

45 Gammon, G. and Sercarz, E. 1989. How some T cells escape tolerance induction. *Nature* 342:183.