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Immunology

Interaction between a "processed" ovalbumin peptide and Ia molecules

(major histocompatibility complex/class II antigens/determinant selection/equilibrium dialysis)

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ABSTRACT **The binding of ^{125}I-labeled immunogenic peptides to purified Ia molecules in detergent solution was examined by equilibrium dialysis. We used the chicken ovalbumin peptide ovalbumin-(323–339)-Tyr, which is immunogenic in the BALB/c mouse and restricted to I-A^d. ^{125}I-labeled ovalbumin-(323–339)-Tyr was shown to bind to I-A^d but not to I-E^d, I-E^k, or I-A^k. This binding was inhibited by unlabeled ovalbumin-(323–339) but not by ovalbumin-(329–339), which is the longest N-terminally truncated peptide that fails to stimulate any of the I-A^d-restricted hybridomas that have been raised to ovalbumin-(323–339)-Tyr. As a further specificity control, we also used the chicken egg lysozyme peptide Tyr-(46–61), which has recently been studied by similar methods [Babbitt, B. P., Allen, P. M., Matsueda, G., Haber, E. & Unanue, E. R. (1985) *Nature (London)* 317, 359–361]. We have confirmed that it bound to I-A^k but not to I-E^k, I-A^d, or I-E^d. Thus, a specific interaction between Ia and antigen that correlates with the major histocompatibility complex restriction was demonstrated, strongly arguing in favor of a determinant selection hypothesis for such restriction.**

The recognition of antigen by T cells involves the corecognition of major histocompatibility complex (MHC) antigens on the surface of accessory cells (1, 2). In the case of T cells of the helper/inducer subset, the MHC antigens involved are the Ia antigens. A further complication to understanding antigen recognition by T cells is the evidence that protein antigens are, in general, physically altered by accessory cells prior to being recognized by T cells (3, 4). *In vitro* studies have shown that proteolytic cleavage, or in some cases denaturation, of a protein can mimic whatever intracellular "processing" events take place within an accessory cell (5, 6). As genetic and biochemical evidence for a single antigen/MHC receptor has accumulated (7), the concept of an Ia–processed antigen complex has gained favor (8); however, although certain functional studies have suggested that such a complex may exist (9–11), it has been very difficult to demonstrate it biochemically.

In previous studies, a dominant immunogenic peptide of chicken ovalbumin that is responsible for 25–35% of the T-cell response to ovalbumin in BALB/c mice has been characterized (12). This peptide encompasses residues 323–339 in the ovalbumin sequence (12). A radioiodinated synthetic peptide with the appropriate sequence was used in an attempt to demonstrate binding of the peptide directly to the relevant MHC restriction element, I-A^d, on the surface of antigen-presenting cells. By use of Ia$^+$ and Ia$^-$ cell lines and monoclonal anti-I-A^d antibodies, no evidence for a specific Ia–antigen interaction was obtained (12). Recently, Babbitt *et al.* (13) have used soluble purified Ia protein in equilibrium dialysis experiments to study its interaction with an immunogenic peptide derived from chicken egg lysozyme. These authors detected binding between the peptide and the relevant restriction element, I-A^k, that did not occur with an irrelevant MHC protein, I-A^d.

The present study was undertaken to determine whether similar methods could be used to demonstrate an interaction between the ovalbumin-(323–339)-peptide and its restriction element, I-A^d.

MATERIALS AND METHODS

Cells. The B-cell lymphoma A20-1.11 (A20) was used as a source of I-A^d and I-E^d. The B-cell lymphomas CH12 and AKTB-1b were used as a source of I-A^k and I-E^k. A20 and CH12 cells were maintained *in vitro* by culture in RPMI 1640 medium supplemented with 50 μM 2-mercaptoethanol and either 5% horse serum (A20) or 5% fetal bovine serum (CH12). AKTB-1b cells were maintained *in vivo* by serial transfer in AKR mice.

Affinity Purification of Ia Molecules. Purification of Ia molecules was carried out as described (14), with minor modifications. I-E^d and I-A^d were purified from Nonidet P-40 (NP-40) lysates of A20 cells by affinity chromatography using the monoclonal antibodies 14-4-4 (I-E^d- and I-E^k-specific) and MK-D6 (I-A^d-specific) coupled to Sepharose 4B (Pharmacia, Uppsala). Similarly, I-E^k and I-A^k were purified from lysates of CH12 or AKTB-1b by affinity chromatography using the monoclonal antibodies 14-4-4 and 10-3.6 (I-A^k-specific). To improve the purity of the Ia preparations, the columns were washed with phosphate-buffered saline (PBS, 0.13 M NaCl/0.02 M phosphate, pH 7.0) containing 0.1% $NaDodSO_4$ and 0.5% NP-40, before elution of the Ia molecules.

Protein Determinations. The BCA (bicinchoninic acid) protein assay (Pierce) was used, with bovine plasma albumin (Bio-Rad) as the standard.

Equilibrium Dialysis. Equilibrium dialysis in 1% NP-40/PBS containing phenylmethylsulfonyl fluoride (1 mM), *o*-phenanthroline (260 μg/ml), pepstatin 150 μg/ml, and EDTA (3 mg/ml) was done in a Hoefer (San Francisco) Microdialyzer EMD 101 with a 100-μl "inside" compartment containing Ia or gelatin (USP grade, Baker) separated from a 250-μl "outside" compartment by a membrane with a nominal molecular weight cut-off of ≈14,000. ^{125}I-labeled peptide was added to both compartments to a final concentration of 40 nM, and dialysis proceeded at room temperature for 48 hr. Preliminary experiments established that this was sufficient time for equilibrium to be reached when the peptide was added to only one compartment. After dialysis, the inside and outside compartments were collected, their volumes were determined by weight, and the amount of radioactivity within each compartment was determined in a gamma spectrometer. The degree of binding of the labeled peptide to the Ia or

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Abbreviation: MHC, major histocompatibility complex.

gelatin is expressed as an "inside vs. outside" ratio (I/O) defined as [(cpm inside/weight inside)/(cpm outside/weight outside)] × 100. Thus, an I/O of 100 signifies the distribution across the membrane of labeled peptide at equilibrium in the absence of binding, whereas an I/O greater than 100 indicates binding. All experiments were performed in duplicate and, of the more than 80 duplicates performed, the standard deviation (paired comparison) was less than 3% of the mean. Binding exceeding an I/O of 106 is significant at the 95% level.

Peptide Synthesis. Peptides were synthesized by the general solid-phase methods outlined by Merrifield *et al.* (15), on a Beckman model 990B or on an Applied Biosystems (Foster City, CA) model 430A peptide synthesizer. Protected peptide intermediates were prepared by using a polystyrene copolymer to which protected amino acids had been esterified either with or without an organic linker. In general, the *t*-butoxycarbonyl group was used for the temporary protection of N^{α} and was removed by treatment with 50% trifluoroacetic acid in CH_2Cl_2, after which coupling was affected by dicylohexylcarbodiimide mediated with 1-hydroxybenzotriazol. When the 430A synthesizer was used, the protected amino acids were first converted to amino acid symmetric anhydrides and the dicyclohexylurea was removed by washing in CH_2Cl_2. The preformed symmetric anhydride was then added to the vessel containing the deprotected resin-bound peptide for coupling. The completed peptide was cleaved from the resin and protecting groups were removed simultaneously by treatment with liquid HF containing 10% (vol/vol) anisole or a combination of 5% (vol/vol) anisole and 5% (vol/vol) dimethyl sulfide. After treatment with HF, the peptide was extracted from the resin with trifluoroacetic acid, the peptide solution was evaporated to 5 ml, and the peptide was precipitated by the addition of ether. The precipitate was collected by filtration, washed with ether, and dried. After preliminary gel filtration with Sephadex G-15, the peptide was purified by HPLC on a Vydac (Hesperia, CA) or Waters Associates C_{18} column by elution with CH_3CN in 0.2% trifluoracetic acid. The purified peptides were analyzed for amino acid composition and amino acid sequence on an Applied Biosystems model 470A gas-phase sequencer. Peptides were uniformly >90% pure by these criteria.

RESULTS

Previous experiments had demonstrated that the tryptic peptide ovalbumin-(323–339) contains an immunodominant region within ovalbumin that is recognized by class II-restricted T cells from BALB/c mice. More than 50 T-cell hybrids that show reactivity with this peptide have been studied, and although several different antigenic determinants appear to exist within this peptide region, T-cell specificity has always been found to be associated with corecognition of I-A^d. We reasoned that if the MHC restriction to the I-A^d subregion was in part due to the affinity of this peptide for the I-A antigen, then binding should be observed with that molecule and not with the I-E^d antigen. Table 1 shows the results of five equilibrium dialysis experiments which used a radioiodinated synthetic ovalbumin-(323–339)-peptide that was synthesized with a tyrosine at the C terminus for the purpose of radiolabeling [ovalbumin-(323–339)-Tyr. I-A^d and I-E^d were isolated from the A20-1.11 B-cell lymphoma and were used at the same molar concentration (which varied from 12–46 μM) in the experiments shown. Although the binding to I-A^d varied somewhat from experiment to experiment, in all experiments there was significant binding of the ligand to I-A^d that was not observed with I-E^d nor with the irrelevant protein gelatin, which was used at the same weight concentration as the Ia antigens. The standard deviations of replicate samples in these experiments did not exceed 3% of the mean and thus, even the lowest degree of binding observed (i.e., 110% in experiment 4) was significantly higher ($P < 0.05$) than that observed with I-E^d.

Table 1. ^{125}I-labeled ovalbumin-(323–339)-Tyr binds to I-A^d but not to I-E^d

	I/O				
	Exp. 1 (28 μM)	Exp. 2 (46 μM)	Exp. 3 (30 μM)	Exp. 4 (30 μM)	Exp. 5 (12 μM)
I-A^d	145.7	142.6	127.3	110.7	113.8
I-E^d	100.6	102.0	100.9	100.7	99.5
Gelatin	—	98.8	98.3	—	102.6

I-A^d or I-E^d (concentration indicated in parentheses for each experiment) was placed in one compartment of an equilibrium dialysis chamber. ^{125}I-labeled ovalbumin-(323–339)-Tyr (40 nM) was added to both compartments. After dialysis for 2 days, the concentration of ^{125}I in each compartment was determined. Data are expressed as the ratio (I/O) of the concentration of ^{125}I in the Ia-containing compartment (inside) to that in the non-Ia-containing compartment (outside) multiplied by 100 (see *Materials and Methods*). Each value represents the mean of duplicate samples. An equal-weight amount of gelatin was included as an additional specificity control in experiments 2, 3, and 5.

Next, the relationship between I-A^d concentration and binding to the peptide was studied (Table 2). At the highest concentration obtainable, we were able to achieve >2-fold higher concentration of ligand on the I-A^d-containing side of the dialysis chamber than on the other side. That the binding of peptide to I-A^d is saturable is shown in Table 3, experiment A; unlabeled peptide was capable of completely inhibiting the binding of labeled peptide to I-A^d when added in 1000-fold excess over the labeled peptide. A truncated peptide, ovalbumin-(329–339), was also used as an inhibitor of the binding of labeled ovalbumin-(323–339)-Tyr to I-A^d. Of all the hybridomas specific for the ovalbumin-(323–339)-peptide, none has been found that reacts with ovalbumin-(329–339), whereas reactivity has been observed to the less-truncated peptides ovalbumin-(327–339) and -(325–339). Even a 1000-fold excess of ovalbumin-(329–339) failed to inhibit the binding of labeled ovalbumin-(323–339)-Tyr to I-A^d (Table 3, experiment B), strengthening the hypothesis that binding of the peptide to I-A^d is relevant for antigenicity.

In a previous study utilizing this type of system, Babbitt *et al.* (13) found that a chicken egg lysozyme peptide representing residues 46-61 in the lysozyme molecule, was capable of binding to I-A^k but not to I-A^d. We decided, therefore, to include I-A^k in our investigation of the binding of the ovalbumin peptide and to synthesize the lysozyme peptide, with an N-terminal tyrosine, Tyr-{lysozyme-(46–61)}. I-A^k and I-E^k were isolated from the H-2^k B-cell lymphomas AKTB-1b and CH12 by similar procedures used to isolate Ia^d antigens. In experiments in which the ovalbumin peptide and the lysozyme peptide were compared with one another for binding to either Ia^d or Ia^k antigens, no affinity of the ovalbumin peptide for I-A^k or I-E^k was observed, nor was there any binding of the lysozyme peptide to I-A^d, I-E^k, or I-E^d (Table 4). Thus, for these two immunogenic peptides, the only significant binding occurred to the Ia antigen for

Table 2. I-A^d concentration-dependence of binding of ^{125}I-labeled ovalbumin-(323–339)-Tyr

I-A^d, μM	I/O*
73.7	209.8
36.8	154.6
18.4	136.8

*See *Materials and Methods*.

Table 3. Specificity of binding of ^{125}I-labeled ovalbumin peptide to I-A^d

Exp.	Inhibitor	Conc., μM	I/O
A	None	—	142.6
	Ovalbumin-(323–339)	0.4	132.5
		4	113.3
		40	101.8
B	None	—	112.6
	Ovalbumin-(323–339)	40	103.7
	Ovalbumin-(329–339)	40	112.5

The concentration of ^{125}I-labeled ovalbumin-(323–339)-Tyr was 0.04 μM. In experiment A, increasing amounts of unlabeled ovalbumin-(323–339)-peptide led to increased inhibition of binding of the labeled peptide; complete inhibition was obtained with 1000-fold excess unlabeled peptide. Experiment B shows the failure of the nonstimulatory peptide ovalbumin-(329–339) to inhibit the binding of ^{125}I-labeled ovalbumin-(323–339)-Tyr to I-A^d. Gelatin and I-E^d controls were performed in both experiments and gave I/O values that ranged from 98 to 102.

which a known MHC restriction has been observed. It should be noted that in our experiments with the H-2^k haplotype, immunization with ovalbumin has never led to the detection of T cells that recognize the ovalbumin-(323–339)-peptide.

DISCUSSION

This study has documented the affinity of an immunogenic peptide derived from chicken ovalbumin for I-A^d, which is not shared by three other Ia molecules, I-E^d, I-E^k, and I-A^k. This binding was detectable by a modification of the equilibrium dialysis procedure described by Babbitt *et al.* (13). In our study we used radioiodinated peptide, in contrast to the fluorescence-labeled probe that these other workers used. In preliminary experiments, we determined that the synthetic ovalbumin peptide that contained iodinated tyrosine at the C terminus had similar functional activity, with respect to the amount of peptide required to stimulate a peptide specific T-cell hybrid, as that of the non-tyrosinated peptide. Similar data are not available for the lysozyme peptide, and it is possible that iodination had some effect on the function of this peptide with respect either to its binding to Ia antigens or to its capacity to stimulate appropriately primed T cells. However, from a qualitative standpoint the pattern of binding was similar to that described by Babbitt *et al.* The actual affinity of the ovalbumin peptide for I-A^d situated in the membrane of a live accessory cell is unknown and may differ significantly from the affinity observed in our equilibrium dialysis experiments. In particular, the nature of the Ia present in the detergent solution is not known. That is, if multiple Ia molecules are present in a mixed micelle with detergent, there may be cooperative interactions between the Ia molecules which would affect the apparent equilibrium constant. Bearing these caveats in mind, it is still useful to compare the apparent affinity of the ovalbumin peptide for I-A^d with the affinity reported by Babbitt *et al.* (13). If one assumes a simple equilibrium between the ovalbumin peptide and monomeric I-A^d molecules, then an apparent equilibrium constant of approximately 2×10^{-6} M was observed, which is similar to that reported for the lysozyme peptide and I-A^k. However, the data obtained with ovalbumin suggest that not all I-A^d molecules were capable of interacting with the peptide. Whether this was due to denaturation of the I-A^d molecules upon preparation by affinity chromatography or due to structural heterogeneity of I-A^d that allows only some of the I-A^d molecules to participate in the interaction with the peptide is not known.

Table 4. Capacity of ovalbumin-(323–339)-Tyr and Tyr-{lysozyme-(46–61)} to bind I-A^k and I-A^d

	I/O					
	Ovalbumin-(323–339)-Tyr			Tyr-{lysozyme-(46–61)}		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
I-A^d	*113.8*	*137.3*	*110.6*	103.1	—	102.4
I-E^d	99.5	—	—	101.4	—	—
I-A^k	—	98.7	99.3	—	*151.3*	*168.0*
I-E^k	—	—	101.2	—	104.3	101.7
Gelatin	102.6	98.1	100.9	100.5	100.0	101.7

In three experiments, various combinations of Ia antigens and ^{125}I-labeled ovalbumin peptide or lysozyme peptide were studied by equilibrium dialysis. Significant binding occurred only between the ovalbumin peptide and I-A^d and between the lysozyme peptide and I-A^k (I/O values in italics). In each experiment, equimolar amounts of each Ia antigen were used: experiment 1, 12 μM; experiment 2, 12 μM; experiment 3, 20 μM.

Our findings, together with those of Babbitt *et al.* (13), provide direct evidence in support of a determinant selection model of immune-response (Ir) gene control (16, 17) and suggest that a major factor in determining MHC restriction is the affinity of an immunogenic peptide for an Ia molecule. An alternative theory of Ir gene control, which states that nonresponsiveness results from "holes" in the T-cell repertoire rather than a failure in antigen–Ia interaction, is supported by studies that demonstrate the capacity of "nonresponder" accessory cells to present antigen to responder T cells (18, 19). Since within the ovalbumin-(323–339)-peptide at least five different antigenic determinants exist, as defined by reactivity of T-cell clones to truncated and homologous peptides, it is likely that the observed nonresponsiveness to this peptide in the *k* haplotype is caused not by multiple holes in the T-cell repertoire, but rather by the inability of this peptide to associate with I-A^k molecules.

However, it is likely that both mechanisms may be involved in determining the responder status of an animal of a particular MHC haplotype to protein antigens. The binding of a processed antigen to an Ia molecule is likely to be an essential prerequisite but may in some cases be an insufficient determinator of MHC restriction, since either holes in the T-cell repertoire or T-cell suppression may dictate functional nonresponsiveness, thus overriding the possibility of a response created by the antigen–Ia interaction. As more peptide antigens are studied in this way, we anticipate the appearance of examples of peptides that bind to a particular Ia molecule but nonetheless cannot be presented functionally to T cells on that Ia background. Information on the antigen–Ia interaction should be important in terms of elucidating the mechanisms by which nonresponder status occurs in such cases.

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