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Immunology

Relative contribution of "determinant selection" and "holes in the T-cell repertoire" to T-cell responses

(antigen–major histocompatibility gene complex interaction/immune response/major histocompatibility gene complex restriction)

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ABSTRACT **Using BALB/c and CBA/J mice, the *I*-region associated (Ia) binding capacity and T-cell immunogenicity of a panel of 14 overlapping peptides that span the entire sequence of the protein staphylococcal nuclease (Nase) was examined to evaluate major histocompatibility gene complex (MHC) control of T-cell responses. Ia binding and Ia-restricted T-cell immunogenicity could be determined for a total of 54 peptide–MHC combinations. Only 30% of the 54 instances examined involved detectable Ia binding, but they represented almost all (12 of 13) of the immune responses found. However, binding to Ia was not sufficient to ensure T-cell immunogenicity, since only 70% of the binding events were productive—i.e., were associated with an immune response. Thus, Ia molecules have the expected characteristics of a highly permissive capacity for antigen interaction that allows them to function as restriction elements for a large universe of antigens. On the other hand, since the Ia molecules cannot distinguish between self and non-self, not all antigen–Ia interactions would be permitted to elicit a T-cell response. It appears that both Ia binding ("determinant selection") and T-cell repertoire act in concert to define the immune response status of an individual toward any particular T-cell epitope.**

Major histocompatibility complex (MHC) molecules exert a considerable control on T-cell responses (1). In general, T cells will only recognize antigen displayed in association with self-MHC molecules ("MHC restriction"). Furthermore, T cells from a given strain of inbred animal will or will not be able to recognize a particular antigen depending on the MHC haplotype of the animal ("responder status"). Broadly, two main theories have been proposed to explain this MHC control of T-cell responses. According to one line of reasoning ("determinant selection"), MHC molecules act as specific antigen receptors, thereby allowing some, but not all, antigens to interact with a particular MHC and form potentially T-cell stimulatory moieties (2). Thus, only antigenic determinants specifically bound to MHC molecules would be presented to T cells. The other line of reasoning ("hole in the T-cell repertoire") claims that MHC control of T-cell responses is at the level of the T cell itself and not at the level of interaction between antigen and MHC (3). According to this hypothesis, no specific interaction occurs between antigen and MHC. Instead, T-cell specificities for certain antigen–MHC combinations do not exist in the T-cell repertoire of that animal. A prime reason for these functional defects in the T-cell repertoire would be the necessity to delete/suppress potentially self-MHC/self-antigen reactive T cells. In the broadest sense, such holes in the T-cell repertoire may be due to either an absence of the relevant T-cell receptor (caused by limitations of the T-cell receptor gene repertoire or by deletion of T cells) or, alternatively, the unresponsiveness of antigen-specific T cells (caused by anergy or by suppression) (4).

Recently, we and others (5, 6) have been able to demonstrate that peptide and MHC interact specifically, thereby providing strong evidence for the existence of determinant selection. Using a panel of 12 immunogenic peptides and four different MHC class II (Ia; *I*-region associated) molecules, we found a very good correlation between the capacity of an Ia molecule to bind a particular antigen and the capacity of that Ia to serve as a restriction element for T-cell recognition of the antigen (7). This ability to measure the formation of antigen–MHC complexes has made it possible to evaluate the extent to which determinant selection influences the immune response to a foreign protein. In this paper, we have used a panel of 14 overlapping peptides representing the entire sequence of the M_r 16,800 protein staphylococcal nuclease (Nase) (8), to estimate the frequency of Ia binding sites within an antigen and to evaluate the relative contribution of determinant selection vs. putative holes in the T-cell repertoire in the generation of T-cell responses. We found that only ≈30% of the peptide–Ia combinations studied were capable of forming potentially immunogenic peptide–Ia complexes, and, of these, only ≈70% could actually elicit a T-cell response. The remaining instances of detectable Ia binding, but not T-cell immunogenicity, are presumably caused by holes in the T-cell repertoire. In only a single instance did a peptide with low to undetectable Ia binding capacity elicit a T-cell response. These findings further substantiate the proposed role of Ia in T-cell-mediated immune responses in which Ia molecules have a specific, though broadly permissive, capacity to bind peptides derived from protein antigens. Thus, the selection by Ia molecules of antigen determinants for presentation is probably an absolute requirement for T-cell immunogenicity, whereas other factors, presumably the repertoire of the T cells themselves, ultimately determine whether or not the antigen–MHC complex will be immunogenic.

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, and the AKTB/1b lymphoma was used as a source of I-A^k and I-E^k. These cells were grown and cell lysates were prepared as described (7).

Affinity Purification of Ia Molecules. I-A^d, I-A^k, I-E^d, and I-E^k molecules were purified as described (7), using the

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Abbreviations: Nase, staphylococcal nuclease; MHC, major histocompatibility gene complex; APC, antigen-presenting cell; Ia, *I*-region associated.

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monoclonal antibodies MKD6 (I-A^d specific), 14-4-4 (I-E^d and I-E^k specific), and 10.3.6 (I-A^k specific) coupled to Sepharose 4B beads.

Ia–Peptide Binding Assay. Purified Ia molecules (10–40 μM) in a detergent solution [phosphate-buffered saline (PBS) with 2.6% digitonin (Sigma) for I-$A^{d/k}$ or with 0.05% Nonidet P-40 (NP-40) for I-$E^{d/k}$] containing a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride/1.3 mM phenanthroline/73 μM pepstatin A/8 mM EDTA/6 mM *N*-ethylmaleimide/200 μM 7-amino-1-chloro-3-tosylamido-2-heptanone) were incubated for 2 days at room temperature with $\approx 10^5$ cpm of ^{125}I-radiolabeled peptide (0.2–0.6 μM) and a dose range (1–600 μM) of unlabeled Nase peptide. The resulting peptide–Ia complexes were separated from free peptide by gel filtration on a Sephadex G50 (Pharmacia) column (1.5 × 22 cm). The columns were eluted in 0.5% NP-40/PBS/0.1% NaN_3 and collected in 1-ml fractions, which were assayed for radioactivity by γ spectrometry. The fraction of peptide bound to Ia (α; typically $\approx$10%; range, 5–40%) relative to the total amount of offered peptide was calculated as the ratio of peptide found in the void volume to the total amount of peptide recovered. The α was corrected by subtracting the α value obtained when labeled peptide was incubated with 600 μM unlabeled peptide in the absence of Ia (typically $\approx$1%; range, 0.1–2%). In previous experiments, using the same Ia batch on consecutive days, the standard deviation was found to be <5% of the mean α.

All experiments were performed a minimum of three times. The concentration of unlabeled peptide needed to inhibit the binding of the labeled peptide to Ia by 50% was determined (7). In this series of experiments, using several batches of Ia over a long period, a 3-fold or more difference of the mean 50% inhibition dose between any of the peptide–Ia combinations reported is significant at the 95% level. The sensitivity of the assay depends on the concentration of unlabeled peptide used, which frequently depends on solubility. In the reported experiments, 600 μM was the highest concentration of unlabeled peptide used.

Peptide Synthesis. The peptides used for radiolabeling were synthesized as described (7). The Nase peptides were synthesized by the solid-phase method. The *t*-butoxycarbonyl (Boc)-amino acid coupled to hydroxymethylphenyl acetaminodomethyl (Pam)-polystyrene was purchased from Applied Biosystems. The peptide assembly at a 0.5-mmol scale was carried out automatically with an Applied Biosystems 430A peptide synthesizer, using Boc-amino acids (Peninsula Laboratories) to give 14 protected peptidyl resins containing peptides whose sequences correspond to the sequence of Nase (8). Specifically, Nase peptides containing residues 1–20, 11–30, 21–40, 31–50, 41–60, 51–70, 61–80, 71–90, 81–100, 91–110, 101–120, 112–130, 121–140, and 131–149 were prepared. After treatment of each peptidyl resin with anhydrous hydrogen fluoride (10 ml per g of resin) in the presence of *p*-cresol (1 ml per g of resin) and *p*-thiocresol or 1,2-ethanedithiol (1 ml per g of resin) for 1 hr at 0°C, the resins were washed with diethyl ether (20 ml, five times) to remove scavengers and with 30% glacial acetic acid (20 ml, five times) to solubilize the synthetic peptides. The peptides recovered from the aqueous phase were desalted and purified as described (9). Aliquots of each peptide were hydrolyzed in 6 M HCl containing 0.2% phenol at 110°C for 24 hr, and the hydrolysates were analyzed with a 6300 amino acid analyzer (Beckman). The observed compositions of the peptides corresponded to the theoretical compositions. Tryptophan, destroyed by acid hydrolysis, was not detected. Glutamine and asparagine, deamidated during acid hydrolysis, were detected as glutamic acid and aspartic acid. Fast atom bombardment mass spectrometry of the purified peptides, carried out with a JMS-HX-110 (JEOL) identified the expected $(M + H)^+$ for each of the peptides.

T-Cell Proliferation Assays. BALB/c, CBA/J, and B10A-(4R) mice were bred at the National Jewish Center Animal Facility and were $\approx$8 weeks old when used. Mice were immunized with 25 μg of each of the different Nase peptides subcutaneously at the base of the tail in complete Freund's adjuvant. Ten days later, the animals were sacrificed, and the inguinal and para-aortic lymph node cells were harvested. T cells were purified by passage over nylon wool (10). In triplicate microtiter plates, 4×10^5 T cells per well were incubated with 2×10^5 (4000 rad, γ-irradiated; 1 rad = 0.01 Gy) spleen cells, together with 1 μg of peptide. [^{3}H]-Thymidine incorporation was measured by pulsing with 1 μCi (1 Ci = 37 GBq) during the last day of a 5-day culture. The mean response was averaged, and the background (T cells plus spleen cells without antigen) was subtracted. The restriction element(s) used by the T cells was also determined. For the H-2^k-restricted responses, immunizations of B10.A-(4R) (I-A^k haplotype) animals and CBA/J (I-A^k and I-E^k haplotype) animals are shown. In addition, monoclonal anti-Ia antibody inhibition of presentation was also used to determine the restriction element(s). Monoclonal anti-Ia antibodies (MK-D6 for I-A^d, 14-4-4 for I-$E^{d/k}$, and 40 M for I-A^k) were protein A purified. Antibody (0.1–2 μg) was added to each well at the initiation of the T-cell proliferation cultures, and the percentage of inhibition obtained with these monoclonal antibodies was calculated. These experiments were performed a minimum of three times.

RESULTS

Binding of Nase Peptides to Ia. Fourteen peptides representing the sequence of the 149-amino acid protein Nase were synthesized. Each peptide was 19 or 20 amino acids long and overlapped the sequence of the adjacent peptides by 9 or 10 amino acids. The sequences of the peptides used in this study were identical to those synthesized by Finnegan *et al.* (9), except we replaced Nase-(111–130) with Nase-(112–130). This synthetic design was chosen to optimize the chances of identifying all T-cell determinants within the intact Nase. The size of these peptides is similar to the size of peptides that have been shown to bind to Ia and be immunogenic, and the overlapping nature of the peptides reduces the risk of missing a T-cell determinant due to the arbitrarily chosen division between peptides.

The ability of this set of peptides to bind to the Ia molecules expressed by mice of the *H-2^d* and *H-2^k* haplotypes was measured by their capacity to inhibit the binding of radiolabeled peptides that had been previously characterized for their capacity to bind to particular Ia specificities. Table 1 shows the capacity of four different affinity-purified Ia molecules (I-A^d, I-E^d, I-A^k, and I-E^k) to bind the panel of 14 Nase peptides. Five peptides [Nase-(21–40), -(41–60), -(51–70), -(71–90), and -(131–149)] bound very weakly or not at all to any of the four Ia molecules tested (IC_{50}, >600 μM). One peptide, Nase-(101–120), bound with a 50% inhibition dose of 600 μM or less to all four Ia molecules tested. The remaining eight peptides bound to from one to three Ia molecules with a 50% inhibition dose of 600 μM or less. The binding between peptide and Ia was specific; i.e., a single Ia could not bind all peptides, and with the exception of Nase-(101–120), a single peptide could not bind to all Ia molecules tested. Given the sensitivity level of this assay (IC_{50}, <600 μM), five peptides were found to bind to I-A^d, two to I-E^d, three to I-A^k, and eight to I-E^k. Because of the overlapping nature of the peptides, binding of two adjacent peptides to a particular MHC molecule might be due to a single Ia binding site. If this were the case in all instances of adjacent peptide binding to the same MHC, then there would be three peptide binding regions for I-A^d, one for I-E^d, two for I-A^k, and six for I-E^k.

Table 1. Binding of a panel of 14 Nase peptides to four Ia molecules

Peptide	Concentration of Nase peptide needed to obtain 50% inhibition of binding, μM			
	^{125}I-Ova-(323–339) to I-A^d	^{125}I-λ repressor-(12–26) to I-E^d	^{125}I-HEL-(46–61) to I-A^k	^{125}I-Nase-(101–120) to I-E^k
Nase-(1–20)	25 (4.7)	—	—	300 (1.7)
Nase-(11–30)	150 (2.0)	—	—	450 (2.0)
Nase-(21–40)	—	(±)	—	(±)
Nase-(31–50)	—	(±)	—	500 (2.5)
Nase-(41–60)	—	—	—	—
Nase-(51–70)	—	—	—	—
Nase-(61–80)	225 (2.2)	—	—	500 (2.4)
Nase-(71–90)	—	—	—	—
Nase-(81–100)	(±)	—	—	25 (2.6)
Nase-(91–110)	125 (1.5)	—	65 (1.6)	(±)
Nase-(101–120)	20 (1.2)	500 (2.9)	400 (1.2)	35 (2.8)
Nase-(112–130)	—	150 (1.6)	550	350 (1.9)
Nase-(121–140)	—	—	—	125 (1.8)
Nase-(131–149)	—	—	—	—

Affinity-purified Ia molecules were incubated with a radiolabeled peptide known to bind to that Ia and a dose range of unlabeled Nase peptide. The degree of binding of the labeled peptide to the Ia was determined by a gel filtration assay. Data are expressed as the geometric mean and SD (in parentheses) of the Nase peptide concentrations needed to obtain 50% inhibition of the binding of the radiolabeled peptide to Ia. As positive controls, the concentrations of peptide that led to a 50% inhibition of binding of radiolabeled peptide to Ia were as follows: 7 μM (3.3) ovalbumin (Ova)-(323–339)/I-A^d, 14 μM (3.3) λ repressor-(12–26)/I-E^d, 5 μM (1.8) hen egg lysozyme (HEL)-(46–61)/I-A^k, and 48 μM (1.9) pigeon cytochrome *c*-(88–104)/I-E^k. In these experiments, a 3-fold difference or more between any of the recorded means is significant at the 95% level. —, IC_{50} of >1250 μM; (±), weak binding (IC_{50} extrapolated to 600–1250 μM).

Immunogenicity. We next studied the capacity of each of the 14 Nase peptides to immunize either BALB/c (I-A^d, I-E^d), CBA/J (I-A^k, I-E^k), or B10.A(4R) (I-A^k) mice (Table 2). Most of the peptides were consistently either stimulatory or nonstimulatory. A few peptides [e.g., Nase-(11–30) and Nase-(31–50) for CBA/J] gave inconsistent low positive responses. We, in analyzing the data, have considered a peptide eliciting a mean Δ[^{3}H]thymidine T-cell incorporation of <10,000 cpm to be nonstimulatory (all such peptides induced stimulation indices of <2).

For BALB/c mice, only three peptides [Nase-(1–20), -(61–80), and -(101–120)] were found to be immunogenic. For these peptides, both I-A^d and I-E^d could potentially serve as restriction elements for the T-cell responses. To determine the restriction element(s) used, the capacity of the monoclonal anti-Ia antibodies MK-D6 (anti-I-A^d) and 14-4-4 (anti-I-E$^{d/k}$) to inhibit these T-cell responses was examined. In all instances, the response was inhibited by the addition of MK-D6 to the cultures (80–93% inhibition). In contrast, addition to the cultures of the monoclonal anti-I-E^d 14-4-4 had either no effect [Nase-(1–20)] and Nase-(61–80)] or a partial inhibitory effect [IC_{50}, Nase-(101–120)] on T-cell responses. Thus, all three peptides were restricted by I-A^d, and one of them, Nase-(101–120), was also restricted by I-E^d.

For CBA/J mice (I-A^k, I-E^k), seven peptides [Nase-(1–20), -(11–30), -(81–100), -(91–110), -(101–120), -(112–130), and -(121–140)] were immunogenic. Due to the weakness and inconsistency of the response, the MHC restriction of the response to Nase-(11–30) could not be determined. Of the remaining six immunogenic peptides, three [Nase-(91–110),

Table 2. Immunogenicity of the Nase peptides

Peptide	BALB/c		CBA/J		B10.A(4R)	
	cpm	MHC restriction	cpm	MHC restriction	cpm	MHC restriction
Nase-(1–20)	57 (19–134)	A^d	25 (6–48)	E^k	1 (−1–3)	
Nase-(11–30)	−1 (−5–4)		12 (−6–20)	—	NT	
Nase-(21–40)	3 (−3–9)		4 (−2–10)		NT	
Nase-(31–50)	−1 (−8–3)		9 (−11–31)		NT	
Nase-(41–60)	0 (−1–1)		0 (−3–3)		NT	
Nase-(51–70)	0 (−3–3)		1 (0–1)		NT	
Nase-(61–80)	90 (28–160)	A^d	−1 (−2–2)		NT	
Nase-(71–90)	0 (−1–1)		−2 (−14–5)		NT	
Nase-(81–100)	6 (1–10)		107 (40–131)	E^k	0 (0–1)	
Nase-(91–110)	2 (−2–6)		70 (42–88)	A^k, E^k	33 (30–35)	A^k
Nase-(101–120)	61 (10–138)	A^d, E^d	71 (16–136)	A^k, E^k	27 (20–33)	A^k
Nase-(112–130)	5 (0–13)		110 (31–179)	A^k, E^k	32 (21–42)	A^k
Nase-(121–140)	1 (0–3)		50 (9–101)	E^k	1 (0–2)	
Nase-(131–149)	1 (0–3)		−3 (−6–0)		NT	

Mice were immunized with 25 μg of Nase peptide emulsified in complete Freund's adjuvant. T-cell proliferation was determined by [^{3}H]thymidine incorporation. Shown is the Δ value (×10^{-3}) (the [^{3}H]thymidine incorporation in the absence of peptide subtracted from the incorporation in the presence of peptide). The [^{3}H]thymidine incorporation in the absence of antigen was typically <10,000 cpm. The underlined values indicate immune responses with >10^4 cpm Δ incorporation and a stimulation index of >2. All immunizations were done at least three times, with the exception of the B10.A(4R) responses, which were done twice. The range of the responses is shown in parentheses. Also shown is the Ia restriction as assigned by anti-Ia antibody inhibition studies (data not shown) and for the H-2^k responses by the use of B10.A(4R) (I-A^k haplotype) animals vs. CBA/J (I-A^k and I-E^k haplotype) animals. NT, not tested; —, MHC restriction not determined.

-(101–120), and -(112–130)] were also immunogenic in B10.A(4R) I-A^k mice (Table 2). Thus, combining the immunization results from these two strains, it could be concluded that Nase-(1–20), -(81–100), and -(121–140) were I-E^k restricted, since they were immunogenic in CBA/J and were nonimmunogenic in B10.A(4R). For the other three peptides, Nase-(91–110), -(101–120), and -(112–130), it could be concluded that I-A^k was used as a restriction element, since they were immunogenic in both CBA/J and B10.A(4R) mice. To evaluate whether I-E^k also contributed to the presentation of these three peptides, the capacity of the monoclonal anti-I-$E^{k/d}$ antibody 14-4-4 to inhibit the CBA/J T-cell response of these peptides was examined. Strong inhibition (80–94%) of the response to Nase-(101–120) and -(112–130), and marginal inhibition (49%) of the Nase-(91–110) response was observed. Thus, peptides Nase-(101–120) and -(112–130) were both I-A^k and I-E^k restricted, and peptide Nase-(91–110) was predominantly I-A^k restricted, with a possible I-E^k component as well.

Correlation Between Binding to Ia and Immunogenicity. Finally, the data on the capacity of the four Ia molecules to bind the 14 Nase peptides were combined with the data on the ability of the different Ia specificities to serve as restriction elements for Nase-specific T-cell responses. As previously mentioned, a T-cell stimulation of 10,000 cpm above background was considered significant (stimulation index, >2). The level at which binding of a peptide to Ia was significant with respect to immunogenicity was not known. In Table 3, we have correlated immunogenicity with the relative binding capacity to the peptides to Ia. The binding capacity was divided into strong (IC_{50}, 5–100 μM), intermediate (IC_{50}, 101–600 μM), and weak or undetectable (IC_{50}, >600 μM). Seventeen of the 54 combinations (31%) showed detectable binding to Ia, and a T-cell response was elicited in 12 (70%) of these 17 binding events. Conversely, no T-cell response was elicited in five (30%) of the 17 binding events. With one possible exception [Nase-(91–110) and I-E^k], in no instance did a peptide that did not bind to Ia at this level lead to a T-cell response.

DISCUSSION

T cells recognize protein antigen in a complicated interaction with antigen-presenting cells (APC). These cells do not recognize antigen directly, but only after the antigen has been "processed" (physically altered by denaturation or fragmentation) by an APC (11), and subsequently "displayed" in association with MHC molecules on the APC surface (1, 12). Previous studies on the mechanism of antigen recognition have established that T cells recognize a complex formed between MHC and a peptide antigen (6, 13, 14) and that there is a strong correlation between the capacity of an Ia molecule to bind an antigenic peptide and its capacity to serve as the MHC restriction element used in the immune response to that peptide (5, 7). Although these data indicate that peptide binding to Ia is an important aspect of the immune response, they do not address the impact of such determinant selection in the generation of T-cell responses. What fraction of the universe of peptides can bind to a given type of Ia? Is determinant selection the sole mechanism that dictates whether a peptide will be immunogenic or not, or will other mechanisms such as holes in the T-cell repertoire also operate in defining the immune response to an antigen? The present work has addressed these questions by examining the Ia binding capacity and the immunogenicity of a series of unbiased (not previously selected for being immunogenic) peptides that, in sum, represent the sequence of an entire protein.

Table 3. Correlation between binding to Ia and immunogenicity

Binding to Ia (IC_{50}), μM	Immunogenicity		
	Yes	No	Total
5–100	5	0	5
101–600	7	5	12
>600	1	36	37
Total	13	41	54

In this study, an excellent correlation was found between the capacity of an Ia molecule to bind a peptide and its capacity to serve as the restriction element in the immune response to the same peptide. A weak or undetectable binding was found for 37 (69%) of the total of 54 Nase peptide–Ia combinations examined. With one possible exception that is discussed below, none of these nonbinding events was associated with T-cell immunogenicity. The remaining 17 (31%) showed clear binding between peptide and Ia; 12 of the 17 binding events (≈70% of binding events, ≈20% of all the peptide–Ia interactions examined) were associated with a positive T-cell stimulation, whereas 5 (≈30% of the binding events, ≈10% of all the peptide–Ia interactions examined) did not lead to a T-cell response. This result represents a highly significant ($P < 0.001$) association between the capacity of a peptide to bind to Ia and its ability to use that Ia as a restriction element for T-cell responses.

There are two areas of concern with respect to Table 3 and the interpretation just given above. First, one instance was found in which a peptide with a 50% inhibition dose of >600 μM was capable of eliciting a T-cell response. This involved peptide Nase-(91–110), which elicited a response in CBA/J animals that was predominantly restricted by I-A^k and was also, albeit weakly, restricted by I-E^k. The IC_{50} with I-E^k was >600 μM; however, as indicated in Table 1, a weak binding could be extrapolated between 600 and 1250 μM. Thus, in this case, the T cells might have been sensitive enough to pick up the complexes that the direct binding assay could just barely detect. Alternatively, this may represent an instance in which further processing of the Nase-(90–110) is necessary before binding and immunogenicity are revealed. There are several instances of such a processing requirement even for small peptides (15–18). Another possibility is that this may represent a true exception to the rule of binding at this level being a necessary prerequisite for immunogenicity and would then suggest the existence of an alternative mechanism of generating an immune response besides the "classical" mechanism of forming a peptide–MHC complex. A second complication to the interpretation of the data in Table 3 is that even though the results of the B10.A(4R) immunizations rule out clonal competition for the H-2^k responses, there are two instances in the H-2^d responses in which the assignment of the restriction element used [Nase-(1–20)/H-2^d and Nase-(61–80)/H-2^d] could potentially be complicated by clonal competition, leading to the failure to detect a "cryptic" response restricted to the other Ia isotype. In both these instances, no binding to the other isotype, I-E^d, was detected, and it is in keeping with the main body of data that the nonimmunogenicity of the combinations Nase-(1–20)/I-E^d and Nase-(61–80)/I-E^d was caused by a lack of I-E^d binding rather than by clonal competition. In sum, the correlation between binding to Ia and immunogenicity is highly significant ($P < 0.001$), despite the Nase-(91–110)/I-E^k being assigned as Ia nonbinder and T-cell immunogenic. It remains highly significant, even if the two [Nase-(1–20)/I-E^d and Nase-(61–80)/I-E^d] combinations were to be removed from the correlation because of the potential complication of clonal competition.

The finding of a significant correlation between peptide binding to Ia and immunogenicity strongly supports the determinant selection hypothesis—i.e., that Ia serves as a receptor that selects antigenic determinants and that binding

of antigen to Ia is a prerequisite for T-cell recognition. Assuming that the staphylococcal nuclease peptides are representative of other protein antigens and that physiologically processed antigenic fragments are similar in size to the synthetic peptides we have studied (19), the data would suggest that only ≈30% of the possible peptide–Ia combinations are of an affinity compatible with immunogenicity. That is, at the level of any single MHC specificity, 1/3rd of the potential immunogens are selected for presentation. Furthermore, of the potential immunogens that have been selected on the basis of their capacity to interact with MHC, only ≈70% induced a T-cell response, leaving the other 30% as putative holes in the T-cell repertoire. Thus, both determinant selection and T-cell repertoire act in concert to define the immune responsiveness of an individual. This conclusion is consistent with the finding that Ia molecules do not distinguish between self and non-self (20), which would require holes in the T-cell repertoire to play an important role in maintaining tolerance to potentially immunogenic self-peptide–Ia complexes.

The significance of peptide–Ia interaction in terms of what strength of binding is needed to allow presentation to T cells has previously been unknown. Using a panel of peptides selected for being immunogenic, we found that most of these stimulatory peptides bound to their restriction element with an IC_{50} of 5–100 μM (7). In Table 3, the binding of the Nase peptides to Ia has been divided into three groups (IC_{50} at 5–100 μM, at 101–600 μM, and at >600 μM), and the T-cell stimulatory capacity of these groups was determined. All peptides (5/5) that bound to Ia at an IC_{50} of 5–100 μM were capable of eliciting T-cell responses. Only 7 (58%) of the 12 peptides that bound to Ia at an IC_{50} of 101–600 μM were capable of eliciting T-cell responses, and 1 of 37 (3%) with an IC_{50} of >600 μM elicited an immune response. Thus, the affinity of a peptide for Ia has a profound influence on its T-cell stimulatory capacity. The great majority of the best interactions are productive, dropping off to ≈1/2 of the interactions with IC_{50} of 101–600 μM being productive, and being very rare in those peptides with an IC_{50} of >600 μM. These data suggest that defects in the T-cell repertoire are most pronounced for peptides with intermediate binding affinity for Ia and are rarely seen for the best Ia binding peptides. [Only one peptide, λ repressor-(12–26), has so far been found to bind strongly to a given Ia (*in casu*, I-E^d) without using it as a restriction element in the immune response (7, 21).]

Although the mechanism behind the association of intermediate affinity of MHC binding and the high incidence of putative defects in the T-cell response is unknown, it would be anticipated that a high-affinity interaction would result in larger numbers of MHC–antigen complexes being presented on the surface of an APC, thus inducing high- and low-affinity T-cell clonotypes, whereas lower-affinity interactions would only be expected to result in the stimulation of high-affinity clones. Consequently, defects in the T-cell repertoire would be less likely to be observed for high-affinity antigen–Ia interactions, since a greater number of holes in the repertoire would have to be present for a nonresponder status to be observed.

In our experience, an immunization dose of 10–25 μg of peptide emulsified in complete Freund's adjuvant is sufficient to elicit a T-cell proliferative response against immunogenic peptides. As an implication of the present findings, it becomes of obvious importance to establish whether peptides that exhibit low-affinity binding to Ia and no apparent T-cell immunogenicity can be "rescued" either by higher immunization doses or by multiple immunizations. For these marginally Ia binding peptides, a higher immunization dose could potentially lead to increased formation of peptide–MHC complexes that in turn could activate low-affinity T-cell clones that we might have been unable to detect with our standard immunization protocol. We have conducted preliminary experiments using higher (100 μg) peptide doses with similarly negative results, suggesting that these specificities are, in fact, functionally absent from the T-cell repertoire, although more experiments of this nature are needed to reach a firm conclusion.

On a population basis, Ia molecules are among the most polymorphic proteins known; however, each individual possesses only one or two Ia alleles. Studies of binding of peptides to Ia have suggested that each Ia molecule possesses a single binding site. How does the immune system achieve a sufficient T-cell repertoire despite the requirements for specific binding of antigen to Ia? We have previously found that each Ia can bind many seemingly different peptides and that Ia is very permissive in its capacity to bind antigen, probably because it recognizes broadly defined "motifs" within antigens (1, 7, 16, 22, 23). Indeed, the data presented here demonstrate that of the 14 peptides representing the Nase protein, 5 bound to I-A^d with a 50% inhibition dose of 600 μM or less, 2 to I-E^d, 3 to I-A^k, and 8 to I-E^k. Because 1 binding site might appear in 2 adjacent peptides, a minimum of 3 sites were detected for I-A^d, 1 for I-E^d, 2 for I-A^k, and 6 for I-E^k. Thus, on average, an Ia specificity bound 3 peptide regions within the Nase protein. By extrapolation, it can be estimated that each Ia specificity would bind ≈18 sites on a protein antigen of M_r 100,000. Thus, it does not appear likely that even the smallest microorganism with only a few proteins could escape the immune system due to the absence of Ia binding sites.

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