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Immunology

Antigenic competition at the level of peptide–Ia binding

(antigen presentation/self peptides/histocompatibility molecules)

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ABSTRACT **We examined the direct binding of a hen egg white lysozyme peptide, HEL(46–61), to membrane I-A^k (protein encoded in the *A* locus of the *I* region) molecules in the presence of detergent. A number of synthetic peptide derivatives, which did not stimulate our T-cell reactive hybridomas, competed for the binding of HEL(46–61) to I-A^k and also inhibited the functional presentation of HEL(46–61). Inhibitors included a peptide lacking a tyrosine at position 53 and a peptide corresponding to the autologous lysozyme peptide. Presentation was examined with cells or with supported planar phospholipid membranes bearing only I-A^k and HEL(46–61). Other peptides that did not compete for the binding did not inhibit functional presentation. We concluded that the binding of an immunogenic peptide to I-A is critical for presentation, that the I-A molecule does not discriminate between autologous and foreign related determinants but does recognize structurally different peptides. Our evidence suggests that our immunogenic peptide bears noncontiguous amino acids critical for contact I-A binding interspersed with amino acids critical for interaction with T cells.**

We have demonstrated that a class II histocompatibility protein, an *I*-region associated (Ia) molecule, directly binds to a defined synthetic immunogenic peptide derived from hen egg white lysozyme (HEL) (1). We measured the binding of a fluorescently labeled immunogenic HEL peptide, 4-nitrobenzo-2-oxa-1,3-diazole (NBD)-HEL (46–61), to the I-A^k molecule (protein encoded in the *A* locus of the *I* region), in detergent, by equilibrium dialysis. The peptide HEL-(46–61) contains the immunodominant epitope recognized by mice of the k haplotype (2). The binding was shown to be specific, with a K_d of approximately 2 μM. Furthermore, the binding was haplotype specific, in that NBD-HEL(46–61) did not bind to the I-A^d molecule, a product of a nonresponder allele, when measured under identical conditions. These results may provide necessary insights to understand how Ia molecules can influence T-cell activation in an antigen specific manner.

We have now addressed the question of whether synthetic peptide derivatives of the immunogenic HEL(46–61) peptide, generated by a series of amino acid deletions, substitutions, or truncations, can compete with NBD-HEL(46–61) for binding to I-A^k using our established binding assay system (1). At the same time we have studied the effect of some of these same peptides on the functional presentation of HEL(46–61) by intact antigen presenting cells or by supported planar phospholipid membranes containing purified I-A^k. Our intention is to probe the functional domains of the immunogenic HEL(46–61) peptide—i.e., what residues are necessary for contacting or binding to the Ia molecule, and what residues are necessary for interaction with the T-cell receptor. Included in our group of peptides is an autologous lysozyme peptide containing a phenylalanine in place of leucine-56, [Gln^{49}, Phe^{56}]HEL(49–61), which allowed us to address the question of whether or not self peptides might compete for presentation with foreign determinants, at the level of the Ia molecule.

MATERIALS AND METHODS

Purification of I-A^k. I-A^k was affinity purified on a large scale using an affinity matrix, the anti-I-A^k monoclonal antibody 10-2.16 coupled to CNBr-activated Sepharose 4B from plasma membranes derived from $\approx 10^{11}$ TA3 hybridoma cells as described (3). Briefly, a plasma membrane-enriched fraction was isolated, washed in high salt (0.5 M NaCl, 10 mM EDTA), lysed in 0.5% Triton X-100 containing 15 mM triethanolamine, 0.2 mM phenylmethylsulfonyl fluoride, leupeptin at 10 μg/ml at pH 8.0, and passed through the anti-I-A^k affinity column. I-A^k was eluted with 0.5% deoxycholate in 0.5 M NaCl, pH 11, into neutralizing buffer [0.5% deoxycholate, 0.5 M triethanolamine, (pH 7)]. As shown by NaDodSO$_4$/PAGE under reducing conditions, the purified preparation contained mainly the 33-kDa (α) and 28-kDa (β) subunits of I-A^k with trace impurities. I-A^k was quantitated by a fluorescamine assay and stored at 0°C until further use.

NBD Labeling of HEL(46–61). HEL(46–61) was labeled at its amino terminus with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole(NBD-F) in 0.15 M sodium borate (pH 9.5) using a 3-fold molar excess of NBD-F. The conjugate was purified by reverse-phase HPLC, identified, and quantitated by characteristic NBD-amino conjugate absorption spectroscopy (4).

Antigen Presentation by Peritoneal Macrophages. The presentation of HEL(46–61) by peritoneal macrophages to 3A9 T-cell hybrids was described (5). 3A9 is a T-cell hybridoma that recognizes HEL(46–61) (2, 5). Briefly, 2×10^5 peptone-elicited peritoneal exudate cells from CBA/J mice previously infected with *Listeria monocytogenes* were allowed to adhere for 2 hr to 96-well microtiter plates (Costar, catalogue no. 3596). Monolayers were subsequently washed to remove nonadherent cells and then fixed with 1% paraformaldehyde. To the macrophage monolayer was added 0.316 μM HEL(46–61) together with various amounts of synthetic peptides to be tested. 3A9 T cells (10^5) were then added, and the level of T-cell stimulation was determined by quantitation of the interleukin 2 (IL-2)-dependent [^{3}H]thymidine incorporation by CTLL cells. It is important to note that the maximal concentration of IL-2 released by the 3A9 cells in a 100-μl aliquot when assayed by the CTLL cells was on the linear portion of the dose–response curve as determined by assessment of various dilutions of the original culture supernatants. Finally, in selected experiments the B-cell hybridoma, TA3, was used as an antigen presenting cell.

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Abbreviations: HEL, hen egg white lysozyme peptide; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; I-A, antigen mapping to the *A* locus of the *I* region; Ia, *I*-region associated; IL-2, interleukin 2.

Antigen Presentation by Supported Planar Membranes Containing Affinity Purified I-A^k. I-A^k was reconstituted with egg phosphatidylcholine and cholesterol (7:2 molar ratio; Avanti Polar Lipids) by detergent dialysis into synthetic phospholipid vesicles. I-A^k (544 pmol) in 400 μl was added to 87.4 nmol, 433 nmol, or 1.3 μmol of egg phosphatidylcholine/cholesterol small unilamellar vesicles in 100 μl at a final deoxycholate concentration of 2%. The resultant clear phospholipid/detergent/protein mixed-micellar solution was filter sterilized through a 0.2-μm pore-size filter and dialyzed against four changes of phosphate-buffered saline (ref. 1, pH 7.4) (2 liters each) at 4°C for 96 hr in a SpectraPOR 2 dialysis membrane. The resultant phospholipid vesicles were finally dialyzed overnight against serum-free medium and stored in sterile plastic test tubes at 4°C until further use.

Planar lipid membranes were prepared by a modification of the procedure of Watts *et al.* (6) in 24-well culture dishes using detergent-washed 12-mm round glass coverslips, and lipid vesicles containing I-A^k. Phospholipid vesicles (100 μl) at 0.2 mM lipid plus 100 μl of serum-free medium were placed in 16-mm wells and immediately overlaid with ethanol-sterilized coverslips. After 30 min of incubation, 3.3 ml of the medium was added, and the coverglass was overturned to expose the planar membranes. Medium (3 ml) was removed and then replaced twice to wash away any unbound materials. Next, 3.2 ml of medium was removed then 200 μl of medium containing 20% (vol/vol) fetal calf serum was added followed by 100 μl of HEL(46–61) in 20% (vol/vol) media. Finally, 4×10^5 3A9 T- cell hybridomas in 10% (vol/vol) fetal calf serum were added and incubated 24 hr at 37°C. Aliquots (100 μl) of supernatants were removed and assayed for [^{3}H]thymidine incorporation. For studies of inhibition by the various peptides a 20-fold molar excess was first added to the planar membranes for 30 min followed by addition of HEL(46–61) and 3A9 cells.

Binding of Peptides to I-A^k in Detergent. The binding of NBD-HEL(46–61) to I-A^k in detergent was measured as described (1). Briefly, 12 nmol of NBD-HEL(46–61) was mixed with 1.2 nmol of I-A^k in 800 μl of 0.5% Triton X-100 in phosphate-buffered saline, pH 7.4, and placed in 6.4-mm diameter SpectraPOR 2 membrane tubing and dialyzed versus 50 ml of the same buffer at 37°C for 3 days. Following dialysis the amount of NBD-HEL(46–61) bound to I-A^k at equilibrium was determined by comparison of fluorescence intensity (excitation wavelength, 465 nm; emission wavelength, 535 nm) inside versus outside the dialysis bag. Identical results were obtained when NBD-HEL(46–61) and the various other peptides tested were initially placed outside of the dialysis bag and then allowed to reach equilibrium binding for the same period of time. Absolute amounts of NBD-HEL(46–61) bound and free were determined by comparison of sample fluorescence to appropriate standard curves as described (1). Five-, 10-, and 25-fold molar excesses of various peptides were premixed with NBD-HEL(46–61) and then incubated with I-A^k itself. Percent inhibition was calculated as [nmol NBD-HEL(46–61) bound − nmol NBD-HEL(46–61) bound in the presence of nonstimulatory peptides]/[nmol NBD-HEL(46–61) bound] × 100.

Peptides. All peptides were prepared as described (5) using the general solid-phase method of Merrifield *et al.* (7). Following synthesis peptides were purified by HPLC on a Vydac C_{18} column using a linear gradient of acetonitrile in water from 0 to 80%. Peptides were quantitated by amino acid analysis and stored at −20°C until used. The following HEL peptides (see Table 1) were used in the present study. HEL(46–61) contains the immunogenic determinant recognized by our T-cell lines, 2A11 and 3A9, used in all antigen presentation experiments. HEL(50–61) is a truncated peptide able to stimulate our T-cell hybrids. HEL(54–61) is a truncated peptide unable to stimulate our T-cell hybrids. Des-Tyr^{53}-HEL(50–61), a deletion (or diiodination) of tyrosine-53 renders the peptide nonstimulatory to our T-cell hybrids. The 3-NO_2-tyrosine derivative stimulates 3A9, but not 2A11, cells indicating that tyrosine-53 is an important contact residue for the T-cell receptor or perhaps exerts some type of critical allosteric effect within the peptide. [Gln^{49}Phe^{56}]HEL(49–61) contains the mouse sequence (autologous determinant) and is nonstimulatory to our T-cell hybrids indicating that leucine-56 is essential in generating the determinant. [Phe^{56}]HEL-(49–61) gives identical results further indicating that glycine-49 is not involved in the formation of the determinant; therefore, these two peptides are used interchangeably. HEL(53–57) and (58–61) are two truncated peptides nonstimulatory to our T-cell hybrids, used to determine if the HEL(46–61) peptide is composed of two laterally separated functional domains—i.e., an amino-terminal T-cell receptor binding domain and a carboxyl-terminal I-A^k binding domain. HEL fibrinopeptide B is an immunogenic peptide presented in context of I-A^k (8). HEL(46–53) (S→K) contains conservative amino acid substitutions at positions 54–61 and is nonstimulatory to our T-cell hybrids. This is the HEL "analogue" in ref. 1.

RESULTS

Competition Studies Using Antigen Presenting Cells. Our previous studies examining the antigen fine-specificity of HEL-specific T-cell hybridomas identified several nonstimulatory peptides that contained single-amino acid substitutions or deletions from the immunogenic peptide HEL (46–61). These peptides included [Phe^{56}]HEL(49–61) and Des-Tyr^{53}-HEL(50–61). These two nonstimulatory peptides were able to inhibit the functional presentation of HEL-(46–61) by fixed macrophages to 3A9 T cells (Fig. 1). Significant inhibition was seen at both the 10-fold and the 31.6-fold molar excess of competitor. The failure of HEL(46–53)(S→K) and fibrinopeptide B to inhibit the response proves that this inhibition is a specific event. Note also that the HEL(53–57) and HEL(58–61) peptides failed to inhibit the response.

Competition Studies Using Planar Lipid Membranes. To rule out the possibility that inhibition by nonstimulatory peptides of HEL(46–61) presentation to 3A9 cells by fixed macrophages was due to some type of binding interaction between the various peptides and some unknown key protein(s) on the antigen presenting cell surface, we tested these same peptides in a system of antigen presentation by planar lipid membranes (6). Two other advantages of this system are that it allows us to more accurately quantitate the inhibition and presentation phenomenon and to show that the inhibition is occurring at the level of the I-A^k molecule, which is the only protein present in this particular functional system.

It was first necessary to show that antigen presentation was indeed a function of the I-A^k:phospholipid molar ratio and that the I-A^k–HEL(46–61) product determined the degree of antigen presentation (9, 10). As shown in Fig. 2 the stimulated IL-2 release from 3A9 cells was a function of the I-A^k density, increasing dramatically as the phospholipid:I-A^k molar ratio decreased from 2400 to 800. Furthermore, antigen presentation was also dependent upon the product of I-A^k density and antigen concentration; increasing the HEL(46–61) concentration from 1 to 10 μM increased the [^{3}H]thymidine incorporation resulting from antigen presentation by planar lipid membranes at the molar ratio of 800 from ≈2000 to ≈7000 cpm and at the molar ratio of 2400 from no presentation to ≈2000 cpm (background, <100 cpm). Finally, we also found that the 10-2.16 monoclonal antibody against the I-A^k β chain completely inhibited antigen presentation and that all three components [phospholipid, I-A^k, and HEL(46–61)] needed

Table 1. HEL peptides

	Amino acid residue																Ability to stimulate
	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	3A9 T cells
HEL(46–61)	Asn	Thr	[ ]	Asp	Gly	Ser	Thr	Asp	Tyr	Gly	Ile	Leu	Gln	Ile	Asn	Ser	Arg	+
Mouse HEL(46–61)	Asp	Arg	Gly	—	Gln	—	—	—	—	—	Phe	—	—	—	—	—	—	−
[Phe^{56}]HEL(49–61)					—	—	—	—	—	—	Phe	—	—	—	—	—	—	−
[Gln^{49}, Phe^{56}]HEL(49–61)				Gln	—	—	—	—	—	—	Phe	—	—	—	—	—	—	−
HEL(49–61)				—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
Des-Tyr^{53}-HEL(50–61)					—	—	—	[ ]	—	—	—	—	—	—	—	—	—	−
HEL(52–61)*							—	—	—	—	—	—	—	—	—	—	—	+
HEL(53–61)								—	—	—	—	—	—	—	—	—	—	−
HEL(54–61)									—	—	—	—	—	—	—	—	—	−
HEL(53–57)								—	—	—	—	—						−
HEL(58–61)													—	—	—	—	—	−
HEL(46–53)(S→K)	—	—	—	—	—	—	—	—	Ser	Val	Phe	Thr	Tyr	Ala	Gly	Lys		−

This table lists all HEL peptides used in this and other published reports (1, 2, 5).
*Shortest peptide able to stimulate our T-cell hybrids, 3A9 and 2A11, indicating that aspartic acid-52 is a critical residue for generation of the immunogenic determinant.

to be present on the coverslip for antigen presentation to occur (data not shown).

Presentation was strongly inhibited by the nonstimulatory Des-Tyr^{53}-HEL(50–61) peptide, as well as by the nonstimulatory self-peptide [Gln^{49}Phe^{56}]HEL(49–61). When used at a 20-fold molar excess the Des-Tyr^{53}-HEL(50–61) and [Gln^{49}Phe^{56}]HEL(49–61) peptides inhibited approximately 80.0%. Neither peptide tested alone was able to stimulate IL-2 secretion from the 3A9 hybrids (data not shown). The other peptides tested in this system, HEL(46–53) (S→K) and fibrinopeptide B, as well as HEL(53–57) and HEL(58–61), were unable to inhibit HEL(46–61) presentation to 3A9 cells even when tested at 50-fold molar excesses.

Competition Studies Using Purified I-A^k in Detergent. The binding of NBD-HEL(46–61) to I-A^k was monitored by detection of its fluorescence signal at 535 nm. The binding was inhibited, as shown in Fig. 3, by a 5- to 25-fold molar excess of native HEL(46–61) up to a level of 87%. In this particular experiment the percent of NBD-HEL(46–61) bound was 8%, or 0.96 nmol, resulting in a k_d of 3.8 μM. HEL(50–61), which is itself stimulatory to 3A9 T cells, showed a high degree of competitive inhibition, 68.0% at a 25-fold molar excess. Interestingly, the HEL(54–61) and Des-Tyr^{53}-HEL(50–61) peptides, although nonstimulatory to 3A9 cells by themselves, also exhibited a high degree of inhibition, 75.9% at a 25-fold molar excess for HEL(54–61), and 60.2% at that same level for the Des-Tyr^{53}-HEL(50–61) peptide. This result indicates strongly that residues 46–53 are not necessary for the binding of HEL(46–61) to the Ia molecule. The [Gln^{49}Phe^{56}]HEL(49–61) substituted peptide, which represents a self-peptide (being identical to the mouse lysozyme sequence), showed strong inhibition of NBD-HEL(46–61) binding to I-A^k in detergent, being 71.3% at a 25-fold molar excess. The HEL(46–53)(S→K) peptide and fibrinopeptide B, which differ to a great degree in their amino acid linear sequence from native HEL(46–61), see Table 1, showed no detectable inhibition even when used at a 25-fold molar excess. In particular, the analog proved that the binding of HEL(46–61) to I-A^k was amino acid-sequence specific, since it is a peptide that differs from HEL(46–61) at positions 54–61 by conservative amino acid substitutions. Finally, neither the HEL(53–57) peptide nor the HEL(58–61) peptide showed any inhibition of NBD-HEL(46–61) binding to I-A^k, even when used at a 40-fold molar excess. In all cases studied, each peptide that was able to inhibit HEL(46–61) binding did so in a dose–response manner. In addition, comparison of the inhibition curve for the immunogenic HEL(46–61) peptide versus the various nonstimulatory or stimulatory truncated peptides indicates a similar binding affinity for all the peptides that bound to I-A^k.

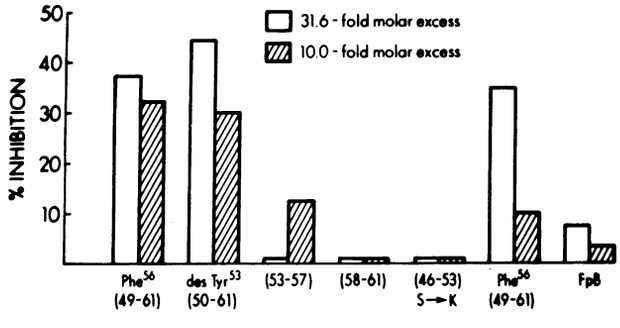

FIG. 1. The inhibition of presentation of HEL(46–61) by fixed macrophages to 3A9 T cells. To a monolayer of fixed macrophages, 0.316 μM of HEL(46–61) was added along with either medium alone or 3.16 μM or 10 μM of the indicated peptides. 3A9 T cells (10^5) were then added and cultured for 18 hr. The level of T-cell stimulation was determined by quantitating the amount of IL-2 released. The % inhibition was determined as follows: % inhibition = [(cpm of medium control) − (cpm with inhibitor)/(cpm of medium control)] × 100. Any negative inhibition values are shown as zero. The data shown were compiled from three separate experiments. The value for the peptide HEL(53–57) at a 10-fold molar excess in the experiment shown, inhibited 12.4%, while the 31.6 molar excess did not inhibit. This peptide, HEL(53–57), has been subsequently tested in three other experiments, and no inhibition was observed at either the 10- or 31.6-fold molar excesses.

DISCUSSION

For a number of peptides, we have found a direct relationship between their capacity to inhibit binding of NBD-HEL(46–61) to I-A^k and their effects on antigen presentation. Only those peptides that inhibited binding reduced antigen presentation. We take this as one more piece of evidence in favor of the concept that an essential feature for immunogenicity of a peptide is for it to bind and interact with the Ia molecule. The experiments using planar lipid membranes containing I-A^k directly established that the site of competition of the various derivatives of HEL(46–61) was the I-A molecule itself. There have been a few reports in the literature concerning the inhibition by nonstimulatory peptides of responses of T cells to immunogenic peptides (11–13). However, in these studies, either the fine structure of the antigen itself has been ill-defined and/or the site(s) of the inhibition event on the presenting cell surface, the Ia molecule, has been inferred but never proven. These studies and our results

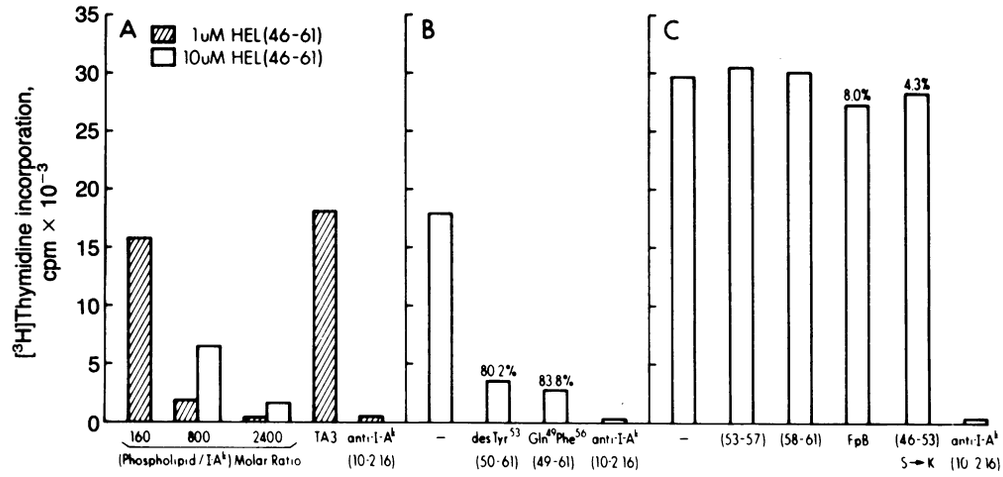

FIG. 2. (*A*) Presentation of HEL(46–61) to 3A9 T-cell hybridomas by planar lipid membranes containing 130 pmol of I-A^k. HEL(46–61) (1 or 10 μM) was incubated at 37°C with 1.6×10^5 3A9 cells and planar lipid membranes of various phospholipid:I-A^k molar ratios. After 24 hr of incubation 100 μl of supernatant was tested for IL-2. Also shown is HEL(46–61) presentation by 1.5×10^5 intact B-cell hybridomas (TA3), and the inhibition of presentation by purified anti-I-A^k monoclonal antibody (10-2.16). (*B*) Inhibition of HEL(46–61) presentation to 3A9 cells by nonstimulatory peptides Des-Tyr^{53}-HEL(50–61) and [$Gln^{49}Phe^{56}$]HEL(49–61) used at a 20-fold molar excess (200 μM). Percent inhibition is indicated above the bar. The response to the presentation of 10 μM HEL(46–61) in the absence of inhibitor (−) by the planar lipid membranes at a phospholipid:I-A^k molar ratio of 800 was 18,065 cpm. (*C*) Inhibition of HEL(46–61) presentation to 3A9 cells by HEL(53–57) and HEL(58–61), and fibrinopeptide B and HEL(46–53)(S→K), all used at 200 μM. The response to the presentation of 10 μM HEL(46–61) in the absence of inhibitors (−) by the planar lipid membranes at a phospholipid:I-A^k molar ratio of 800 was 29,608 cpm. Percent inhibition is indicated above the bar. In all inhibition experiments inhibition by anti-I-A^k (10-2.16) monoclonal antibody of HEL(46–61) presentation to 3A9 cells either by planar lipid membranes or by intact TA3 cells was >90%. In all samples tested the SD were all less than 9% of the sample mean.

leave little doubt that the I-A molecule serves as a site for critical interaction with peptides.

Other critical points to emerge from our data are as follows: (*i*) There was no evidence that HEL(46–61) contained laterally separated functional areas—Ia contact and T-cell receptor contact areas. Neither HEL(53–57) nor HEL(58–61) inhibited presentation—neither peptide inhibited binding of NBD-HEL(46–61) to I-A^k. Heber-Katz *et al.* (14) speculated that an immunogenic peptide contains two discrete binding areas, one for the I-A molecule and another for the T-cell receptor on indirect evidence using cytochromes. Studies with the cytochromes (15) favor results more compatible with our present data. A more realistic model may be one in which Ia contact residues are intermingled with residues that are exposed and available to contact the T-cell receptor. We surmise that some residues—such as the tyrosine-53 and the leucine-56 are not critical for contact to I-A—their absence or modification apparently affects primarily the interaction of the HEL(46–61) peptide with the T-cell receptor. We do not rule out profound changes in the whole structure of the peptide as it interacts with the Ia molecule.

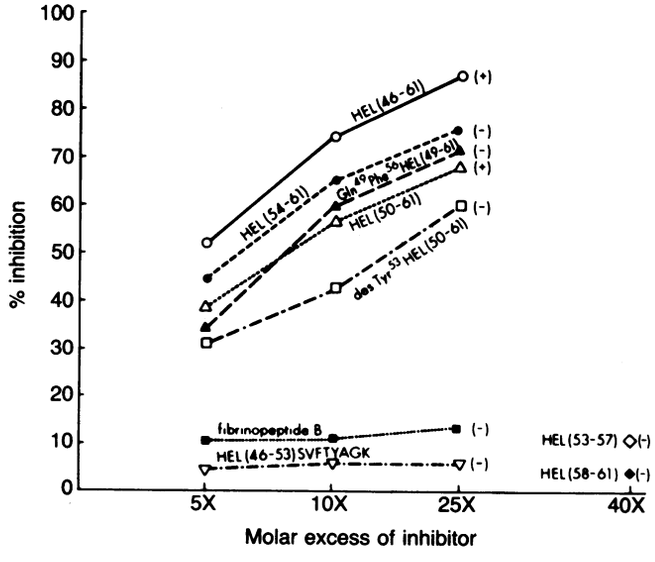

FIG. 3. Inhibition of NBD-HEL(46–61) binding to purified I-A^k in 0.5% Triton X-100 by the indicated peptides (see Table 1). Peptides were preincubated at the indicated molar excesses with NBD-HEL(46–61) and then mixed with I-A^k. After 3 days of equilibrium dialysis at 37°C, sample fluorescence was measured as described (1). The (+,−) designation to the right of each curve indicates whether the peptide is itself stimulatory (+) or nonstimulatory (−) to 3A9 cells.

(*ii*) We have shown that I-A^k in this particular case did not discriminate between a foreign and an autologous antigenic determinant. Although further experimentation is required, we can estimate from our present data that the [$Gln^{49}Phe^{56}$]-HEL(49–61) self-peptide has a K_a for I-A^k that is apparently very similar to that of the immunogenic HEL(46–61) itself. Thus, common/overlapping sites to contact I-A^k exist for the self-peptide and the HEL(46–61) peptide. Consequently, the ability of antigen presenting cell to present foreign proteins to T cells in the presence of great excesses of self-proteins cannot be explained solely from the standpoint of binding affinities (between Ia molecules and peptides) and of multiple binding sites, both phenomena occurring at the cell surface itself. Perhaps only a few foreign peptides–class II histocompatibility ligands or complexes need be present on the cell to be recognized and stabilized by interaction with the T-cell receptor, thereby, transmitting the appropriate transmembrane signal initiating the appropriate activation cascade. This result has far reaching consequences in terms of its implications of the mechanism(s) by which self-proteins and foreign proteins are processed and presented by the various antigen presenting cells; although we believe that for a peptide to be immunogenic, it must first be able to bind to Ia, the binding does not necessarily result in an immune response as exemplified in our study with the autologous HEL peptide. We speculate from our own evidence and that of many others that immune response gene defects would exist, both at the level of I-A binding—as evidenced by the lack of binding of HEL(46–61) to I-A^d (determinant selection) and at the level

of the T-cell receptor (the result with the autologous HEL peptide).

(*iii*) The use of HEL(46–53)(S→K) and fibrinopeptide B gives some idea of the range of specificity of HEL(46–61) for I-A^k. Since fibrinopeptide B is an immunogen presented in the context of I-A^k, it stands to reason that the Ia molecule should have more than one binding site for peptides.

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