Unresponsiveness to a Self-Peptide of Mouse Lysozyme Owing to Hindrance of T Cell Receptor–Major Histocompatibility Complex/Peptide Interaction Caused by Flanking Epitopic Residues

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Summary

A self-peptide containing amino acid residues 46–61 (NRGDQSTDYGIFQINSR) of mouse lysozyme (ML) (p46–61, which binds strongly to the A^k molecule but does not bind to the E^k molecule), can induce a strong proliferative T cell response in CBA/J mice (A^k, E^k) but no response at all in B10.A(4R) mice (A^k, E^o). However, two truncated forms of p46–61, p48–61, or p46–59, are immunogenic in both B10.A(4R) and CBA/J mice. The critical residues within p46–61 reside between amino acid positions 51 and 59. T cells of B10.A(4R) mice primed with the truncated peptides in vivo cannot be restimulated by p46–61 in vitro. This suggests that T cell receptor (TCR) contact (epitopic) residue(s) flanking the minimal 51–59 determinant within p46-61 hinder the interaction of the $p46$-$61/A^k$ complex with the appropriate TCR(s), thereby causing a lack of proliferative T cell response in this mouse strain. Unlike B10.A(4R) mice, [B10.A(4R)×CBA/J]F1 mice responded vigorously to p46–61, suggesting that thymic APC of B10.A(4R) mice do not present a self ligand to T cells resulting in a p46-61–specific hole in the T cell repertoire in B10.A(4R) or the F1 mice. Moreover, APC from B10.A(4R) mice are capable of efficiently presenting p46–61 to peptide-specific T cell lines from CBA/J mice. The proliferative unresponsiveness of B10.A(4R) mice to p46–61 is not due to non–major histocompatibility complex genes because B10.A mice (A^k, E^k) respond well to p46–61. Interestingly, B10.A(4R) mice can raise a good proliferative response to p46–61(R61A) (in which the arginine residue at position 61 (R61) of p46–61 had been substituted by an alanine residue) or equally well to p46–61 (R61L/F/N/K), indicating that R61 was indeed responsible for hindering the interaction of p46–61 with the appropriate TCR. Finally, chimeric mice [B10.A(4R)→B10.A] responded vigorously to p46–61, suggesting that thymic antigen presentation environment of the B10.A mouse was critical for development of a p46–61–reactive T cell repertoire. Thus, we provide experimental demonstration of a novel mechanism for unresponsiveness to a self peptide, p46–61, in the B10.A(4R) mouse owing to hindrance: in this system it is the interaction between the available TCR and the A^k/p46–61 complex, which is hindered by epitopic residue(s) within p46–61. We argue that besides possessing T cells that are hindered by R61 of p46–61, CBA/J and B10.A mice have developed an additional subset of T cells bearing TCRs which are not hinderable by R61, presumably through positive selection with peptides derived from class II E^k, or class I D^k/D^d molecules. These results have important implications in self tolerance, shaping of the T cell repertoire, and in defining susceptibility to autoimmunity.

Tolerance to determinants within a self-protein occurs through several mechanisms. During development of the T cell repertoire in the thymus, the thymocytes that recognize self-peptide/self-MHC complexes with high affinity/avidity undergo clonal deletion owing to apoptosis during the process of negative selection (1–4). In addition, thymic T cell tolerance (central tolerance) can also be induced by nondeletional mechanisms (3, 5). Thymic tolerance can only be induced to those self determinants that are processed and presented most efficiently by the APC in the

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thymic microenvironment. Therefore, additional mechanisms are needed to control the activity of T cells potentially reactive to antigens that are only expressed in the periphery (peripheral tolerance). Several mechanisms have been proposed for induction of peripheral tolerance: (a) mature T cells that recognize a self determinant on a nonprofessional APC in the absence of a costimulatory signal are rendered anergic (6–8), whereas T cells that are stimulated by a high concentration of a peripheral antigen presented by a professional APC can undergo deletion by apoptosis (peripheral deletion) (9–11). (b) The activity of T cells that respond to a self-antigen can be effectively modulated through regulatory or suppressor T cells and cytokines (12–15). (c) Downregulation of the TCR on self-reactive T cells (16) or (d) altered/impaired TCR signaling leading to clonal inactivation, provide additional mechanisms for induction of self-tolerance (17). (e) It has recently been suggested in a transgenic model of spontaneous autoimmunity that peripheral tolerance was a diversion of the differentiation of autoreactive T cells towards Th1 or Th2 effector phenotypes (18). (f) In another case of transgenic mice with autoreactive TCR, self-tolerance was attributed to a defective costimulatory process in transgenic T cells (clonal insufficiency) (19).

In this paper, we report a novel mechanism to explain the lack of response to a self-peptide, p46-61(NRGDQST-DYGIFQINSR), of mouse lysozyme (ML)[1] (20, 21) in B10.A(4R) mice, attributable to hindrance by TCR contact (epitopic) residues in the interaction between the available TCR and the A^k/p46-61 complex. (ML p46-61 had been referred to as p46-62 in previous studies [22–24]. In our earlier study (21) as here, we have numbered the extra glycine residue at position 48 of ML as 47a for easier comparison with the sequence of hen eggwhite lysozyme [HEL]). Our results suggest that T cells reactive to determinant(s) within p46-61 exist in B10.A(4R) mice but these T cells can be activated only by peptides lacking the NH$_2$- or COOH-terminal flanking residue(s) but not by p46-61, and that the above-mentioned hindrance can be relieved by alanine (or L/F/N/K) substitution of a putative hindering residue, COOH-terminal arginine (R61), within p46-61. In this regard, we suggest that other A^k-displaying mouse strains, CBA/J and B10.A, can circumvent this hindrance because of an additional subset of T cells that are unhindered by R61 in p46-61; these T cells were presumably positively selected by that region of H-2^k MHC, which has been deleted in B10.A(4R) mice during the chromosomal recombination event. The results of our experiments using bone marrow chimeras support the above-mentioned hypothesis.

Materials and Methods

Mice. B10.A(4R) (A^k,E^o), CBA/J (A^k,E^k), and B10.A (A^k, E^k) mice were purchased from The Jackson Laboratory (Bar Har-

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[1]*Abbreviations used in this paper:* APL, altered peptide ligand; HEL, hen eggwhite lysozyme; ML, mouse lysozyme; PPD, purified protein derivative.

bor, ME) and bred in our animal facility. [B10.A(4R)× CBA/J]F1 mice were also bred in the same facility. Female mice, 6–16 wk of age, were used in the experiments.

Peptide Synthesis and Purification. Mouse lysozyme (ML) (20, 21) peptides were obtained from the following sources: (a) A peptide containing amino acid residues 48–61 of ML (p48-61) (DQSTDYGIFQINSR) was synthesized in our laboratory using the rapid simultaneous solid-phase multiple peptide synthesis methodology, the so-called "teabag" method, which is based on Merrifield's original solid-phase procedure (25). The peptide was generated on a *tert*-butyloxycarbonyl-amino acid–phenylacetamidomethyl (Pam) resin and its amino acid composition analyzed as previously described (21, 26). (b) Overlapping 15-mer peptides spanning the sequence of ML from amino acid residues 40–68 (TRATNYNRGDQSTDYGIFQINSRYWCNDGK were obtained from Chiron Mimotopes (Clayton, Australia). The peptides were synthesized using the "multi-pin" peptide synthesis technique (27). The terminal amino group of each peptide was acetylated, whereas the carboxy-terminal lysine-proline residues formed diketopiperazine. (c) ML peptides 48–61 (p48-61) and 46–61 (p46-61) (NRGDQSTDYGIFQINSR) were synthesized by Dr. Craig Miles, Macromolecular Resources, Colorado State University, Fort Collins, CO. Manual or automated peptide synthesis was performed using *tert*-butyloxycarbonyl amino acids as described elsewhere (21). (d) ML peptides, p40-53, p42-55, p44-57, p46-59 (NRGDQSTDYGIFQIN), p46-61, p50-64, p52-66, p46-61(R61A) (a peptide having the same sequence as that of p46-61, except that at position 61 there is an alanine in place of the arginine residue), p46-6(R61L), p46-61(R61F), p46-6(R61N), p46-61(R61K), p46-61(N46A), p46-61(R47A), p46-61(R47L), p46-61(N59A), and p46-61(S60A) were synthesized in the UCLA Peptide Core Laboratory directed by Dr. Joseph R. Reeve, Jr., using a multiple peptide synthesizer (396; Advanced Chem Tech, Louisville, KY). The identity and purity of the above-mentioned peptides were determined by fast atom bombardment mass spectrometry. (e) Additionally, a sample of p46-61 was kindly provided by Dr. Luciano Adorini, Roche Milano Ricerche, Milano, Italy.

Lymph Node Proliferation Assay. Mice were immunized with 7–14 nmol (or higher concentrations) per mouse of an ML peptide in PBS, in 1:1 (vol/vol) emulsion with CFA (Difco Laboratories, Detroit, MI), in a hind footpad. After 9 d, the draining lymph nodes were removed, cell suspensions washed twice with HBSS (GIBCO BRL, Life Technologies, Inc., Grand Island, NY), and then cultured in a flat-bottom 96-well plate at a concentration of 5 × 10^5 cells/well in HL-1 serum-free medium (Ventrex Laboratories, Inc., Portland, ME) supplemented with 2 mM L-glutamine, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and different concentrations (1.75–14 μM or higher, final concentration) of the antigen. For the pin peptides, one or two wells were tested per peptide. Tuberculin purified protein derivative (PPD) (Parke-Davis, Morris Plains, NJ) was used at a final concentration of 4 μg/well as a positive control. The cells were incubated with 1 μCi/well of [^{3}H]thymidine (International Chemical and Nuclear, Irvine, CA) for the last 18 h of a 5-d culture. Then, the cells were harvested using a Micro Cell Harvester (Skatron Instruments, Inc., Sterling, VA), and the incorporation of radioactivity was assayed by liquid scintillation counting, using the 1205 Betaplate counter (LKB Instruments, Inc., Gaithersburg, MD). The results were expressed as mean counts per minute of duplicate or triplicate cultures. For presentation of data, background (LNC cultured in medium without antigen) values of cpm were subtracted from the cpm obtained with LNC plus antigen. The final results from a group

of animals immunized with the same peptide were expressed as mean Δ cpm $\pm$ SEM. The results obtained with a peptide synthesized by the teabag method were confirmed with pin peptides as well as peptides obtained from other sources, and vice versa. For some repeat experiments, HL-1 medium supplemented with 1% (vol/vol) heat-inactivated FCS (Gemini Bio-Products, Inc., Calabasas, CA) or X-Vivo 10 serum-free medium (Bio-Whittaker, Walkersville, MD) with or without 2% FCS was used in place of HL-1 serum-free medium. The results of these experiments were comparable to those of the earlier experiments using the same peptides in which HL-1 medium or X-vivo 10 medium without FCS supplementation was used.

Generation and Long-Term Maintenance of T Cell Lines. CBA/J mice were immunized with 7 nmol of p46-61 emulsified in CFA (1:1, vol/vol) in a hind footpad. After 9 d, the draining lymph nodes were removed, single cell suspensions prepared, and washed twice with HBSS. The cells were bulk cultured in a 25-cm² tissue culture flask (Corning Inc., Corning, NY) in 10 ml of Dulbecco's modification of Eagle's medium (DMEM) (Mediatech Inc., Herndon, VA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 5×10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO), and 10% (vol/vol) heat-inactivated FCS in the presence of p46-61. After 2 wk, 6 μl of recombinant human IL-2 (Proleukin, 1 mg/ml stock; Cetus Corp., Emeryville, CA) was added per flask and cells were allowed to grow for another 2 wk. Thereafter, cells were restimulated with irradiated (3,000 rads) syngeneic APC and p46-61. In this way, cells were subjected to several alternating cycles of stimulation with antigen/IL-2 at intervals of 2–3 wk until the requisite number of cells were obtained for testing. The antigen specificity of these T cell lines was tested using a proliferation assay as described below.

Proliferation Assay Using T Cell Lines. T cell lines derived from CBA/J mice, 5×10^4 or 1×10^5 cells/well, were cultured with 3–5×10^5 cells/well of irradiated (3,000 rads) splenic APCs from CBA/J or B10.A(4R) mice in HL-1 serum-free medium as described above, using different concentrations of antigen. There were three sets of controls in the assay: (*a*) APC alone in medium without antigen, (*b*) APC plus T cells in medium without antigen, and (*c*) T cells alone in medium with antigen but no APC. Proliferation of T cells was measured by addition of 1 μCi of [³H]thymidine/well for the last 18 h of a 3-d culture. Incorporation of radioactivity was assayed by liquid scintillation counting as described above. The results were expressed as Δ cpm.

MHC Binding Assays. A^k molecules were purified from detergent-solubilized CH27 (28) B lymphoma membrane preparations using a 10-2-16 mAb immunoaffinity column. Binding assays were performed using a modification of the procedure previously described (29). In brief, purified A^k (50 nM) was incubated with 1 μM biotin peptide and various concentrations of competitor peptides in 30-μl samples containing 0.2% NP-40 and 20 mM citrate/phosphate buffer, pH 5, for 18 h at 37°C. Biotin peptide/MHC complexes were captured on assay plates coated with 10-2-16 mAb. After washing, excess europium streptavidin was added and fluorescence was measured at 612 nm as described (29). Results, representing mean biotin peptide/class II complexes from duplicate samples, are expressed as fluorescence counts per second (cps $\times 10^{-3}$).

Radiation-induced Bone Marrow Chimeras. Chimeric mice were prepared essentially according to the method described elsewhere (30, 31) with a few minor modifications. Briefly, 12–18-wk-old B10.A mice were irradiated with 950 rads using a Co^{60} source (at the Laboratory of Structural Biology and Molecular Medicine,

UCLA). Within 24 h of irradiation, mice were given 1–2×10^7 T cell–depleted bone marrow cells i.v., derived from age- and sex-matched B10.A(4R) mice. The bone marrow cells that were treated with monoclonal anti-Thy 1.2 antibody, J1j.10 (American Type Culture Collection [ATCC], Rockville, MD) and guinea pig complement (GIBCO BRL, Life Technologies, Inc.) in vitro did not reveal any proliferative responsiveness to Con A. Recipients were given neomycin sulphate (GIBCO BRL, Life Technologies, Inc.) (2 g/liter) in their drinking water beginning 10 d before irradiation and continuing throughout the period of experiment. Splenic cells of chimeric mice were stained with two different mAbs separately, 14-4-4S (ATCC) (reactive with the mouse E^k molecule) and KH95 (specific for the mouse D^b molecule) (Pharmingen, San Diego, CA). Isotype-matched mAbs were used as controls and FITC-conjugated appropriate second antibodies were used. Stained cells were fixed with 1% formaldehyde and analyzed by a cytofluorograph (Becton Dickinson and Co., Mountain View, CA). Splenic cells of our chimeras were negative for E^k but positive for D^b, and the staining profile of cells of chimeric mice was similar to that of naive B10.A(4R) mice (data not shown). Chimeric mice were immunized with ML p46-61/CFA for LN proliferation assay (see above) after 10–12 wk of reconstitution.

Results

Response of B10.A(4R) and CBA/J Mice to p46-61 of Mouse Lysozyme. Response to p46-61 of ML was tested in B10.A(4R) (A^k,E^o) and CBA/J (A^k,E^k) mice. This peptide binds strongly to the A^k molecule but not to the E^k molecule (22–24, 32). Mice were immunized in a hind footpad with p46-61 emulsified in CFA, and after 9 d, the draining LNC were tested for proliferative response to the same peptide in vitro. The results are presented in Fig. 1. B10.A(4R) mice did not give any proliferative response to p46-61, whereas CBA/J mice responded vigorously to the same peptide. B10.A(4R) mice did not respond even when very high concentrations (30–50 nmol) of p46-61 were used for immunization (data not shown).

T Cells from CBA/J Mice Primed with p46-61 In Vivo Also Respond to Certain Overlapping Peptides Spanning the Region 40–68 of ML In Vitro. CBA/J mice were immunized with p46-61/CFA, and after 9 d the response of the draining LNC was recalled in vitro using different ML peptides at three concentrations each: 0.7, 7, and 14 μM. The results of a representative experiment using 7 μM of each peptide are given in Fig. 2. These results demonstrate that the critical amino acid residues within the ML determinant that is comparable to HEL p46-61 (33) is very different: the ML core determinant is 51-59, whereas the HEL core determinant is 52-61.

Response of CBA/J and B10.A(4R) Mice to the Truncated ML Peptides, p48-61 and p46-59. To determine the importance of amino acid residues at the NH_2 or COOH terminus of the longer peptide, ML p46-61, in binding to the MHC or in interaction with the TCR, peptides lacking residues 46, 47, and 47a (p48-61) or 60 and 61 (p46-59) of p46-61 were tested for their immunogenicity in CBA/J and B10.A(4R) mice using an LN proliferation assay. It is clear from the results shown in Fig. 3 that, like p46-61,
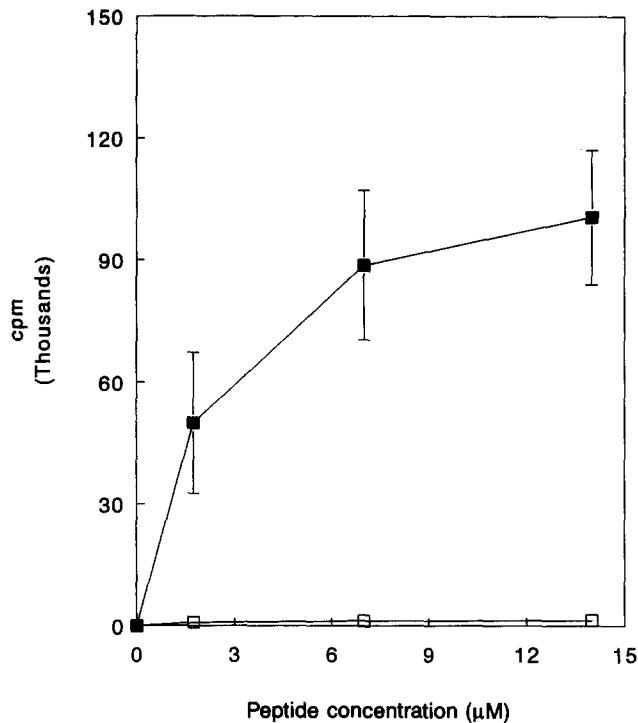
Figure 1. CBA/J (■–■) but not B10.A(4R) (□–□) mice respond to ML peptide 46-61 (NRGDQSTDYGIFQINSR). Mice were immunized in a hind footpad with p46-61 of ML emulsified in CFA, and after 9 d, the response of the draining LNC was tested using a T cell proliferation assay. The results from a group of six animals of each mouse strain were expressed as mean Δ cpm ± SEM. Similar results were obtained on repeat experiments. Identical patterns of proliferative responses were observed with three different preparations of p46-61 (see Materials and Methods). PPD responses of CBA/J and B10.A(4R) mice were comparable in magnitude, and ranged from 92,030 to 246,290 cpm.

p48-61 and p46-59 each induced a strong proliferative T cell response in CBA/J mice. Thus, it is evident that all three peptides can bind optimally to the A^k molecule, and that immunogenicity of the determinant(s) within p46-61 was not affected by the absence of certain amino acid residues within p46-61.

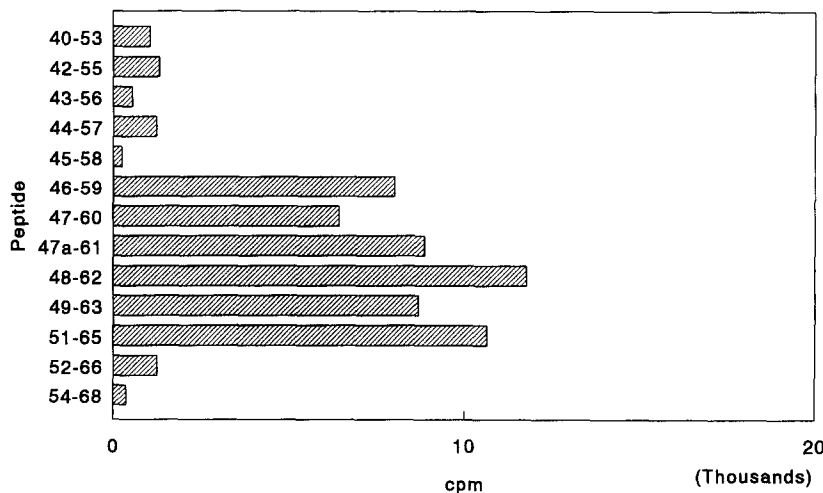Surprisingly, B10.A(4R) mice, which did not respond at

all to p46-61, produced potent proliferative T cell responses to p48-61 or p46-59 (Fig. 3). These results demonstrate that T cells potentially reactive to the core determinant within p46-61 exist in B10.A(4R) mice. However, T cells primed with the above truncated peptides in vivo could not be stimulated in vitro with p46-61 (data not shown), suggesting that the interaction of these T cells with p46-61 is not efficient enough to induce a proliferative T cell response to this peptide. As discussed below, we attribute this lack of response to p46-61 to epitopic hindrance.

Amino Acid Residues at the NH₂ or COOH Terminus of ML p46-61, Including Arginine Residues (R47/R61) Are Not Critical for Binding to A^k. To define further the function of amino acid residues at either terminus of p46-61, we tested the A^k binding of variants of p46-61 prepared by substituting one amino acid at a time with an alanine residue. The following peptides were tested in the assay: p46-61(N46A), p46-61(R47A), p46-61(N59A), p46-61(S60A), and p46-61(R61A). The results given in Fig. 4 show that A^k binding of each of the above five peptides was comparable to that of the native peptide, ML p46-61. Overall, the binding affinities of variant ML peptides and ML p46-61 were within one log of the positive control, HEL p46-61. These results demonstrate that amino acid residues at the NH₂ or COOH terminus of ML p46-61, including terminal arginines, R47 or R61 are not critical for the binding of this peptide to the A^k molecule. Therefore, in the A^k/p46-61 complex, the above-mentioned residues are most likely projecting outward toward the TCR and thus could hinder the interaction between the A^k/p46-61 complex and the TCR.

[B10.A(4R)×CBA/J]F1 Mice Raise Vigorous Proliferative Responses to p46-61. To address further the mechanism underlying the unresponsiveness of B10.A(4R) mice to p46-61, we studied the response of [B10.A(4R)×CBA/J]F1 mice to p46-61. We reasoned that if the lack of response of B10.A(4R) mice to p46-61 were due to a hole in the T cell repertoire caused by expression of a self-peptide/A^k complex in the thymus during development of the T cell repertoire, then the same ligand should also lead to a similar hole in the T cell repertoire in the case of [B10.A(4R)×CBA/J]F1
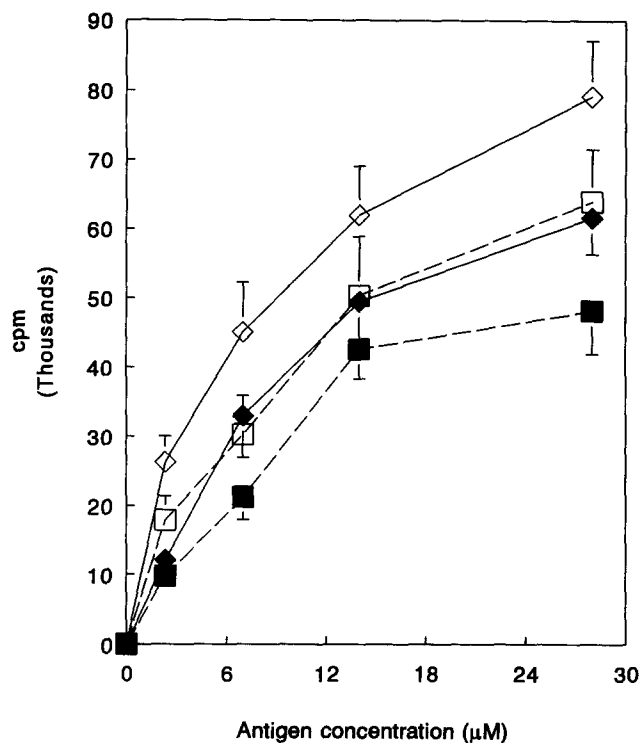

Figure 2. The core of the determinant within ML p46-61 in CBA/J mouse is 51–59, as peptides 46-59 through 51-65 are positive. Mice were primed with p46-61 and 9 d later the draining LNC were examined for response to an almost complete set of overlapping 15-mer peptides spanning the region 40–68 of ML (TRATNYNRGDQSTDYGIFQINSRY-WCNDGK; G following R47 has been numbered as 47a [21]) using 7 μM of each peptide. The results of a representative experiment expressed as Δ cpm are shown in the figure. Similar results were obtained on repeat experiments. Medium alone (cells cultured in medium without antigen) gave 1,498 cpm.

Figure 3. Both CBA/J and B10.A(4R) mice respond to ML peptides 48-61 (DQSTDYGIFQINSR) (CBA/J [◇–◇], B10.A(4R) [◆–◆]) and 46-59 (NRGDQSTDYGIFQIN) (CBA/J [□–□], B10.A(4R) [■–■]). Mice were immunized in a hind footpad with one of the ML peptides emulsified in CFA, and 9 d later, the proliferative response of draining LNC was measured. For each mouse, the peptide that was used for immunization was also used for the in vitro recall response. The results from four animals of each mouse strain immunized with p48-61 or p46-59 are shown as mean Δ cpm ± SEM.

mice rendering them completely unresponsive to p46-61. As shown in Fig. 5, F1 mice raised a potent proliferative response to p46-61, and the level of response was comparable to that of CBA/J mice.

APC from B10.A(4R) Mice Can Present p46-61 to T Cell Lines from CBA/J Mice. We tested the ability of splenic APCs of B10.A(4R) mice to stimulate T cell lines specific for p46-61 derived from CBA/J mice (see Materials and Methods), using a proliferation assay. The results presented in Fig. 6 demonstrate that B10.A(4R) APC can efficiently present p46-61 to the peptide-specific T cells, and that the level of proliferation using B10.A(4R) and CBA/J APC was comparable. Thus, the lack of response to p46-61 in B10.A(4R) mice is not due to a defect in antigen presentation by APC of this mouse strain. Obviously then, the defect must lie at the level of the T cell.

Ala (or Leu/Phe/Asn/Lys) Substitution of Arg-61 or Arg-47 Renders p46-61 Immunogenic in B10.A(4R) Mice. From the above results, we predicted that epitopic residues within p46-61, most likely the bulky arginine residue(s) at position 61 (R61) and/or 47 (R47), were responsible for hindrance in the TCR-p46-61/A^k complex interaction in B10.A(4R) mice. Therefore, we examined the immunogenicity of p46-61(R61A) (in which R61 was substituted by an alanine residue) as well as p46-61(R61L), p46-61(R61F), p46-
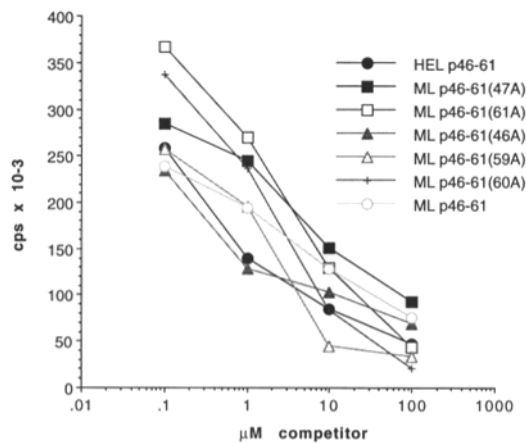

Figure 4. Relative binding affinities of ML p46-61 variants for A^k. Purified A^k (50 nM) was incubated with 1 μM biotin-HEL p46-61 in the presence of competitor peptides at the indicated concentrations at pH 5 for 18 h at 37°C. Bound biotin peptide was quantified using a europium fluorescence immunoassay as described in Materials and Methods. The fluorescence signal in the absence of competitor was 275 cps × 10^{-3}. Results are representative of two experiments.

61(R61N), p46-6(R61K) and p46-61(R47L) in B10.A(4R) mice. These results given in Figs. 7 and 8 show that B10.A(4R) mice can raise strong proliferative responses to p46-61(R61A) (Fig. 7) and to each of the other R61/R47-substituted peptides tested, but not to native p46-61 [=p46-61(R61)] (Fig. 8). Interestingly, B10.A(4R) mice immunized with p46-61(R61F) (*A*) gave the best recall response with p46-61(R61L) and vice versa (*B*). However, in
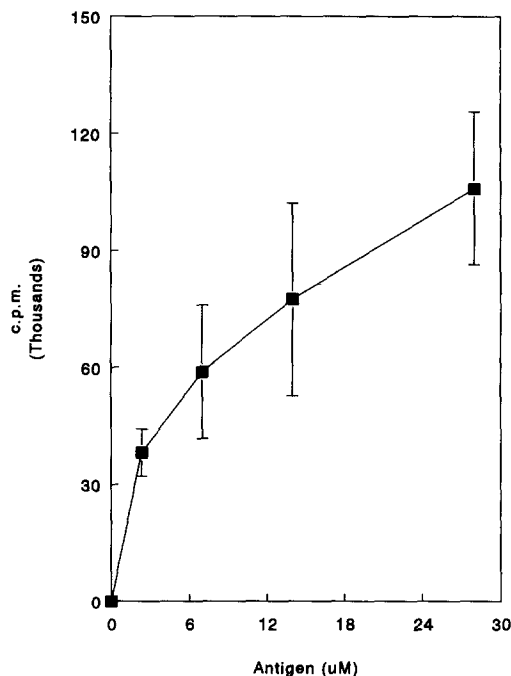

Figure 5. [B10.A(4R)×CBA/J]F1 mice raise vigorous proliferative responses to p46-61. Mice were immunized with p46-61/CFA in a hind footpad and 9 d later, the draining LNC were tested using a T cell proliferation assay. The results from a group of four mice were expressed as mean Δ cpm ± SEM. Similar responses were obtained on repeat experiments.
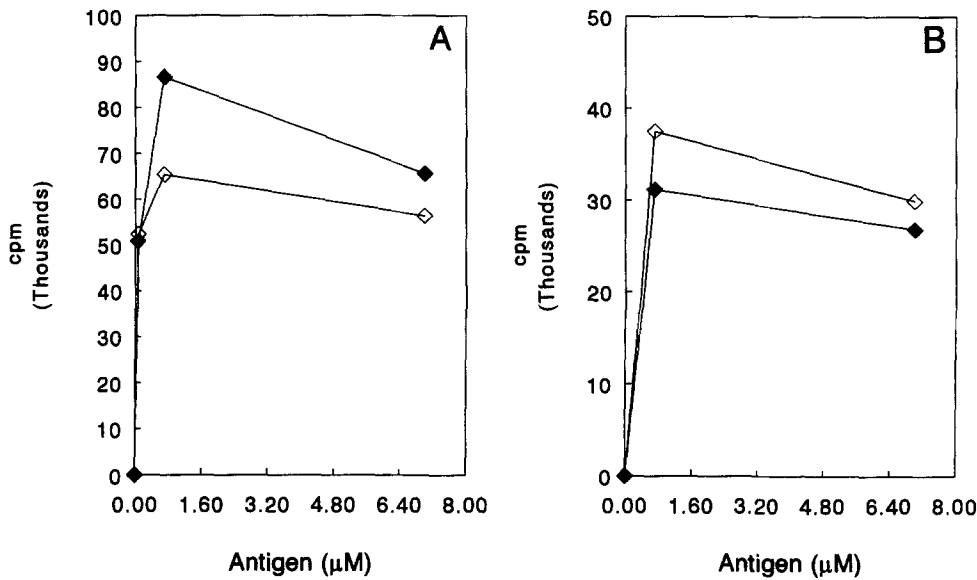
Figure 6. APC from B10.A(4R) mice can efficiently present p46-61 to T cell lines derived from CBA/J mice. Two p46-61–specific T cell lines, CBAML61.1 (*A*) and CBAML61.2 (*B*), both derived from CBA/J mice (see Materials and Methods) were used in a proliferation assay using irradiated (3,000 rads) APC from CBA/J (◆–◆), or B10.A(4R) (◇–◇) mice in the presence of different concentrations of p46-61. The level of cpm obtained with T cells cultured with p46-61 but without added APC was not higher than that of T cells plus APC cultured in medium without antigen (data not shown). The results of a representative experiment are given as Δ cpm. Similar results were obtained in repeat experiments.

both instances, there was no appreciable recall response with p46-61(R61N). In summary, the above results clearly demonstrate that R61 or R47 was indeed responsible for the above-mentioned hindrance.

Response of B10.A Mice to ML p46-61. To determine the role of B10.A background genes in the unresponsiveness of B10.A(4R) (A^k, E°) mice to p46-61, we studied the immu-
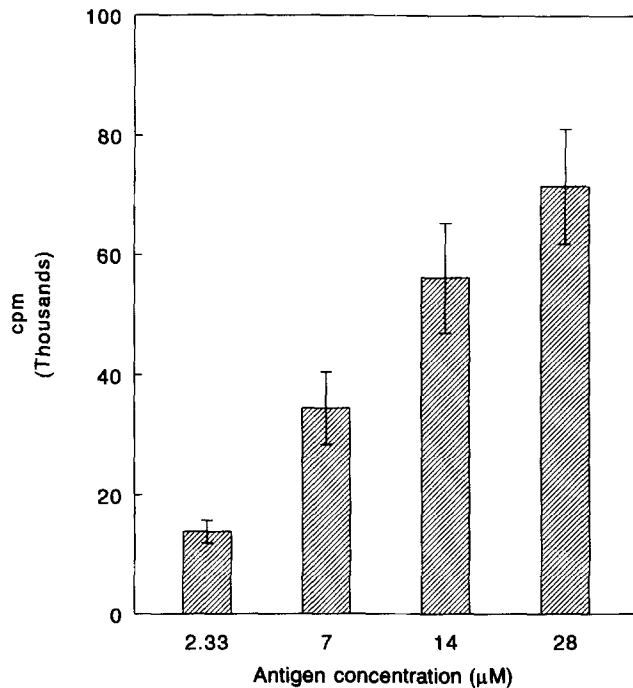

Figure 7. p46-61(R61A) is immunogenic in B10.A(4R) mice. Mice were immunized with a peptide, p46-61(R61A) emulsified in CFA, having an alanine residue in place of the arginine residue at position 61 of p46-61. The proliferative response of draining LNC was measured after 9 d. The results from four mice are given as mean Δ cpm ± SEM. Like B10.A(4R) mice CBA/J mice also gave a significant proliferative response to p46-61(R61A), and the level of response in the two mouse strains was comparable (data not shown).

nogenicity of p46-61 in B10.A mice (A^k, E^k). Surprisingly, p46-61 induced a strong proliferative response in B10.A mice (Fig. 9). Thus, the lack of response to this peptide in B10.A(4R) mice in contrast to responsiveness of CBA/J mice to the same peptide cannot be attributed to differences in non-MHC genes of these two strains.

Radiation-induced Chimeric [B10.A(4R)→B10.A] Mice Respond to p46-61. To test our hypothesis that the subset of T cells unhindered by R61 within p46-61 is positively selected only in B10.A and CBA/J mice but is lacking in B10.A(4R) mice, we prepared radiation-induced bone marrow chimeras. In these chimeric mice, irradiated B10.A mice were reconstituted with T cell–depleted bone marrow cells from B10.A(4R) mice (see Materials and Methods for details). 10–12 wk after irradiation, chimeric mice were immunized with p46-61/CFA in a hind footpad. After 9 d, the draining LNC were tested for proliferative responses to p46-61. The results given in Fig. 10 demonstrate that chimeric mice could raise vigorous proliferative responses to p46-61 (whereas B10.A(4R) mice did not [Fig. 1]). In the same series of experiments, unmanipulated B10.A mice immunized with p46-61/CFA raised good proliferative responses to p46-61, and the magnitude of their proliferative responses was comparable to that of the chimeric mice (data not shown). Thus, the T cell repertoire of B10.A(4R) mice lacked T cells that could respond to p46-61. However, under the thymic microenvironment provided by B10.A mice in chimeric mice, bone marrow T cell progenitors from B10.A(4R) mice whose TCR could potentially accommodate p46-61/A^k complex could be positively selected.

Discussion

In previous studies, p46-61 of ML has been successfully used in B10.A(4R) and C3H.HeJ (A^k, E^k) mice to inhibit in vitro T cell responses to A^k-restricted (but not E^k-restricted) determinants within foreign antigens through MHC block-
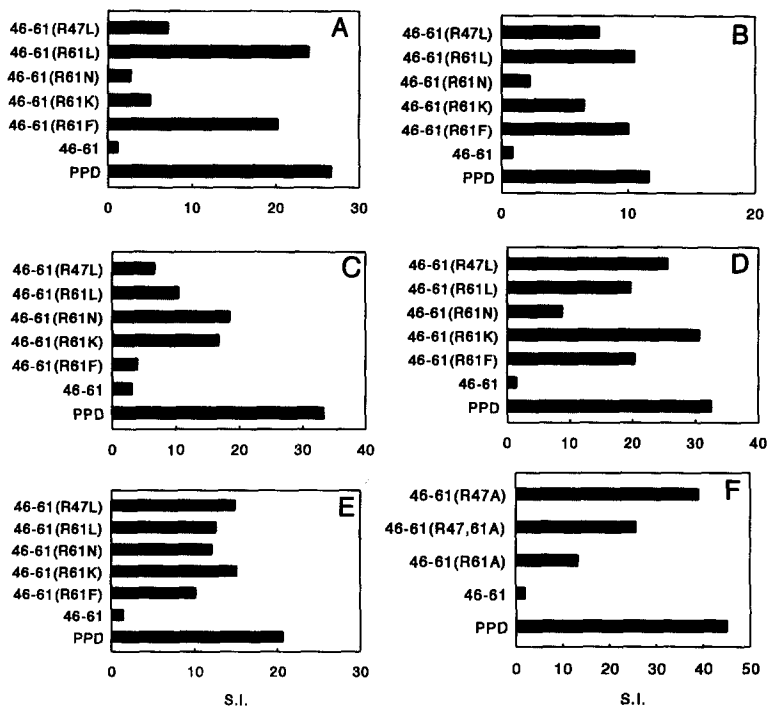
Figure 8. Responses of B10.A(4R) mice to variants of p46-61 having the arginine residue at positions 61 or 47 substituted with one of the following: Phe(F), Leu(L), Asn(N), or Lys(K). Mice were immunized with one of the variants of p46-61 (*A*) R61F; (*B*) R61L; (*C*) R61N; (*D*) R61K; (*E*) R47L; and (*F*) R47A, and the proliferative T cell response in each case was recalled in vitro with different variants of p46-61 or with native p46-61. The results were expressed as stimulation indices (SI = cpm with antigen/cpm without antigen). The results of a representative experiment are shown in the figure. Similar results were obtained in repeat experiments.
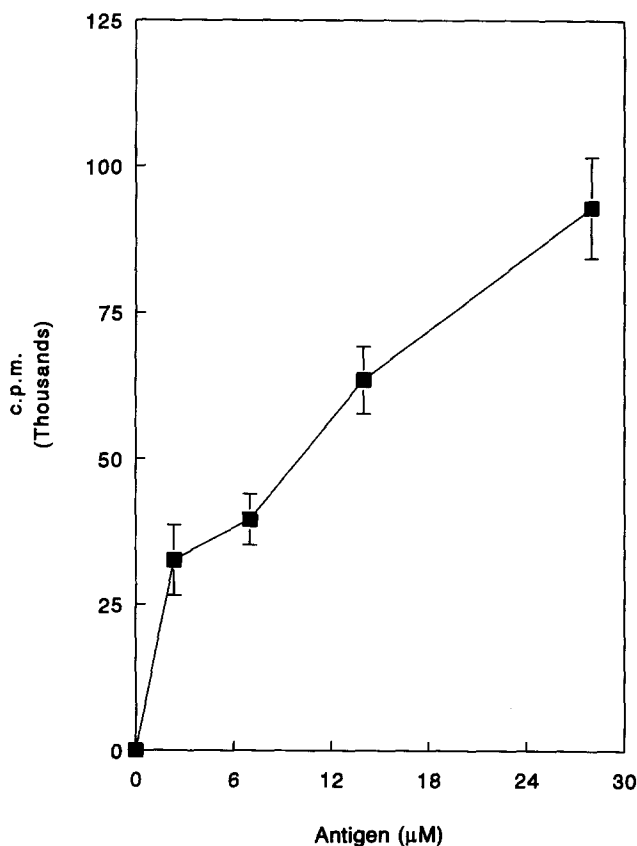
ade (22–24). Furthermore, it was shown that ML p46-61 was nonimmunogenic in the above strains of mice (22, 23, 34). Interestingly, our results demonstrate that other A^k-displaying mouse strains, CBA/J and B10.A, have the ability to respond to p46-61. Since all the above strains of mice presumably share an identical A^k molecule, the unresponsiveness of B10.A(4R) mice to p46-61 could be attributable to: (*a*) a complete hole in the T cell repertoire, whereby T cells bearing TCR potentially capable of responding to all determinant(s) within p46-61 were absent from the T cell repertoire, (*b*) a defect in presentation of p46-61 by APC of B10.A(4R) but not CBA/J mice attributable to differences in non-MHC genes, or (*c*) the existence of T cells potentially reactive to determinant(s) within p46-61, but only activatable by p46-59 or p48-61; however, owing to hindrance, their interaction with the p46-61/A^k complexes on B10.A(4R) APC could not generate a T cell response.

Our results from two sets of experiments do not support the possibility of a complete hole in the T cell repertoire of B10.A(4R) mice for T cells potentially reactive with the determinant(s) within p46-61: (*a*) p48-61 and p46-59, lacking amino acid residues at either the NH$_2$ or COOH terminus of p46-61, respectively, can generate valid T cell responses, indicating that T cells reactive against the immunogenic determinant(s) within p46-61 with a core of 51-59, do exist in this mouse strain. However, these T cells can only be recruited by p48-61 or p46-59 but not by p46-61, which has extensions at either end. As discussed below, we attribute this inefficient interaction to hindrance caused by epitopic residue(s) within p46-61, most likely at the bulky arginine residues, R47 and R61. (*b*) [B10.A(4R)×CBA/J]F1 mice raised a potent proliferative response to p46-61. If

Figure 9. B10.A mice can respond to p46-61. Mice were immunized with p46-61/CFA in a hind footpad, and 9 d later, proliferative responses of draining LNC were tested. The results from a group of four animals are presented as mean Δ cpm ± SEM.
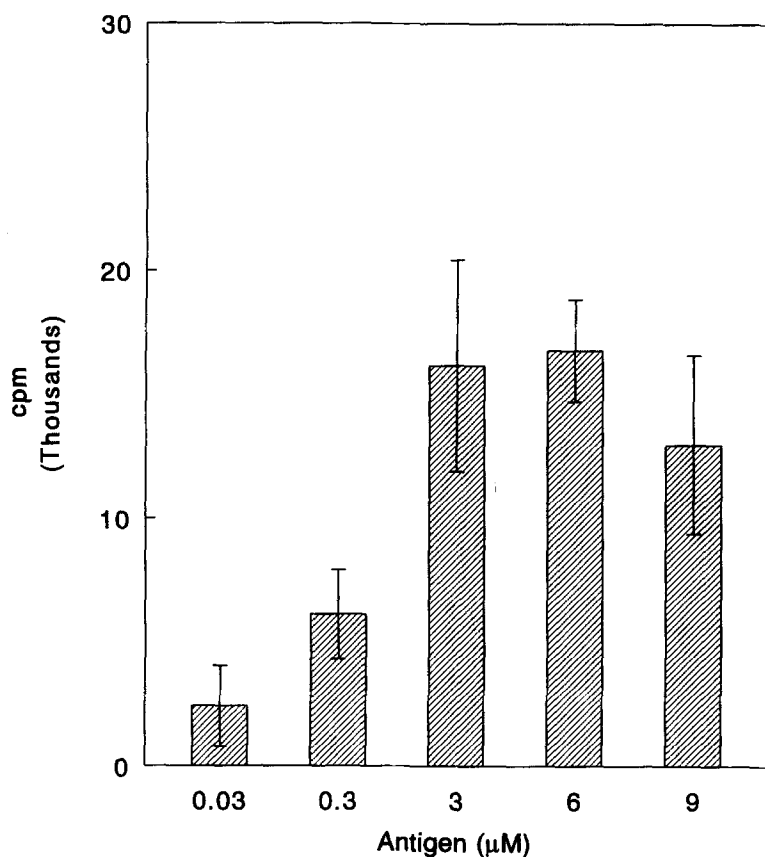
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Figure 10. Radiation-induced bone marrow chimeric [B10.A(4R)→B10.A] mice respond to p46-61. Chimeric mice were immunized with p46-61/CFA in a hind footpad. After 9 d draining LNC were tested in a proliferation assay. The results from a group of four animals are presented as mean Δ cpm $\pm$ SEM.

there were a p46-61–specific hole in the T cell repertoire of B10.A(4R) mice caused by a self-peptide/A^k complex, then the same ligand should also have caused a defect in the T cell repertoire of the F1 mice. On the contrary, if the responsiveness to p46-61 were determined by a particular subset of T cells which are unhindered by R61 as described below in the case of CBA/J and B10.A mice, then the deficiency of these T cells in B10.A(4R) mice should be relieved by the CBA/J parent. Unresponsiveness to an antigen owing to a hole in the T cell repertoire of F1 mice caused by tolerance induced by a self ligand expressed in one of the parents has been previously described in other systems (35, 36), and in both these cases the unresponsiveness was dominant in nature. Thus, in this study the responsiveness of the F1 mice to p46-61 is attributable to T cell clones owing their existence to CBA/J parental genes in the F1.

The unresponsiveness of B10.A(4R) mice to p46-61, in contrast to responsiveness of CBA/J mice to the same peptide, cannot be attributed to defective presentation of antigen to T cells by B10.A(4R) APC due to differences in non-MHC genes of the two mouse strains. Two sets of data support the above conclusion. First, APC from both B10.A(4R) and CBA/J mice could efficiently present the immunogenic determinant within p46-61 to p46-61–specific T cells raised in CBA/J mice. Furthermore, at a given concentration of p46-61, the level of proliferation induced by APCs from the above two mouse strains was comparable. Second, B10.A mice having the same A^k molecule and

background genes as B10.A(4R) mice raised a robust response to p46-61.

The crux of the above observations is that B10.A(4R) mice have T cells with TCR that can interact efficiently either with p46-59 or p48-61, in which extensions at one or the other end of the core determinant exist, but cannot interact with p46-61, which has extensions at both ends. Considering the fact that p46-61 can bind strongly to the A^k molecule, a logical conclusion from these results is that the presence of additional amino acid residues at the NH_2 and/or the COOH terminus in p46-61 cause steric hindrance in the interaction between TCR of T cells reactive to the truncated peptides, p46-59 or p48-61, and the A^k/p46-61 complex. In view of the long arginine side chains at positions 47 and 61, we tested the idea that they were likely candidates for causing this hindrance. Substitution of R61 either by an alanine residue or by L, F, N, or K in p46-61 renders each of the variant peptides immunogenic in B10.A(4R) mice. Similarly, p46-61(R47L) (in which R47 had been substituted with a leucine residue) and p46-61(R47A) were also found to be immunogenic in B10.A(4R) mice. (It is interesting that in H-2^b mice, we have shown that arginine-61 of HEL p46-61 prevents interaction of this peptide with the MHC class II molecule A^b [37].) In any event, although B10.A(4R) TCR can manage to respond with a single hindering residue in place, but not both, they do not possess those T cells that are unhindered by R61 and R47, whereas these T cells exist in CBA/J and B10.A mice as discussed below.

The term "hindering structures" was first proposed by Brett et al. (38) to explain the lack of response to a determinant within equine myoglobin. In that study, it was suggested that a product of natural processing of equine myoglobin probably had hindering structures in the flanking residues of the determinant that interfere with presentation by I-A^k but not by the I-A^s molecule. A similar phenomenon of inhibition/stimulation of T cell responses by amino acid residues either flanking the minimal determinant or at a site some distance away from it, has been described by other investigators (39–43). However, in the above-mentioned studies, although negative interference by flanking residues was assumed for the peptide interaction with Ia or TCR, there was no experimental demonstration that the synthetic peptide with its new extensions, or the naturally processed determinant from the native antigen, either was actually incapable of binding to the appropriate MHC molecule (agretopic hindrance), or of interaction with the TCR (epitopic hindrance). To the best of our knowledge, we believe that our results represent the first complete experimental demonstration of the phenomenon of epitopic hindrance. Recently we have found that an HEL disulfide peptide can bind to A^k but fails to stimulate T cells in the B10.A mouse and this may have a similar explanation (Ametani, A., and E. Sercarz, unpublished results). This same type of finding had been given the same suggestive explanation earlier in the staphylococcal nuclease system by Liu et al. (42). Some recent evidence favors the model that in class II MHC interactions, the unfolding antigen binds and is subsequently trimmed (44, 45). We postulate that steric epitopic hindrance will be a frequent occurrence during the process of trimming unfolded protein fragments. The dangling ends, during and even after trimming, are likely to affect the elicitation of the TCR repertoire, hindering full TCR access to the MHC–Ag complex until the correct processing step occurs. Sometimes, as in this case, a rather small peptide such as ML p46-61 is left bound to MHC class II and retains hindering residues that can influence the access of T cells, either during positive (activation) or negative (tolerance) interactions.

We suggest that the differential response of B10.A(4R) and CBA/J (or B10.A) mice to p46-61 is due to a subtle difference in the T cell repertoire of these strains. The above three mouse strains share a subset of T cells that are reactive to the truncated peptides, p46-59 and p48-61, but the interaction of the TCR on these cells with the p46-61/A^k complex is hindered by epitopic residues within p46-61. However, CBA/J and B10.A mice possess an additional subset of T cells bearing TCR that can accommodate the p46-61/A^k complex and efficiently respond to it. Since B10.A(4R) mice lack the E^k molecule, and also have a recombined MHC gene segment from mice of the H-2^b haplotype, we suggest that the responsiveness of CBA/J and B10.A mice to p46-61, or the lack of it, in B10.A(4R) mice would map within the MHC region from near the class II E^k gene rightward to the position of chromosomal recombination beyond the class I D or L regions or even slightly beyond the MHC into the class Ib region. Accord-

ingly, one or more peptides from a molecule coded for in this region such as E^k (46) or D^k/D^d could have positively selected a ML p46-61–responsive subset of T cells in the CBA/J or B10.A strain which could not have been selected in the B10.A(4R) strain. The T cells thus positively selected may be the ones which are unhindered by R61 and R47 and are recruited by the A^k/p46-61 complex in CBA/J and B10.A mice. The unresponsiveness of B10.A(4R) mice to p46-61 could be explained by the absence of this particular subset of T cells. In this relative sense, at best there is only a mini-hole in the T cell repertoire of B10.A(4R) mice. Our results with bone marrow chimeric mice validate the above-mentioned hypothesis. Nevertheless, we predicted that T cells available in the T cell repertoire of B10.A(4R) mice could have raised a proliferative response to p46-61 even in the absence of this additional subset of T cells described above for CBA/J and B10.A mice, provided there were no hindering residue(s) within this peptide. Our results with truncated peptides lacking certain NH$_2$- or COOH-terminal residues and those of the R61/R47-substituted peptides, each of which is immunogenic in B10.A(4R) mice, provide experimental support for the above prediction.

In this report we have described that p46-61 of ML presented by the A^k molecule to T cells from two different strains of mice can give rise to entirely different outcomes: a proliferative T cell response in the case of CBA/J mice but unresponsiveness in B10.A(4R) mice, presumably due to a subtle difference in the T cell repertoire of these two mouse strains. It is alternatively possible that p46-61 does make limited contact with B10.A(4R) TCR: (a) p46-61 could induce a Th1 response in CBA/J mice but a Th2 response in B10.A(4R) mice. However, data from our preliminary studies do not support this alternative explanation. After immunization with p46-61 in a hind footpad and 9 d later, restimulation of draining LNC with p46-61 in vitro, T cells of CBA/J mice produced high amounts of IFN-γ, whereas those from B10.A(4R) produced very low levels of IFN-γ comparable to that by cells cultured in medium without antigen (negative control) (data not shown). In contrast, neither CBA/J nor B10.A(4R) cells produced IL-4 in amounts above that produced by cells with medium alone. (b) p46-61 of ML could behave as an agonist for TCR from CBA/J mice but function like an antagonist (47) for TCR from B10.A(4R) mice. Thus, there could be differential signaling by the same MHC/peptide ligand after TCR–MHC/peptide interaction in the two mouse strains. In this regard, p46-59 and p48-61 could be categorized as agonists for both B10.A(4R) and CBA/J mice. (c) p46-61 might be considered an altered peptide ligand (APL) (48, 49) in comparison to the truncated peptides, p46-59 and p48-61. In this context, the truncated peptides elicit good proliferative responses in B10.A(4R) and CBA/J mice, whereas the APL, p46-61, induces a differential response in the two strains. However, since the core determinant within p46-61 resides within residues 51–59, it is less likely that residues external to this core would create an APL. Experiments to define further the precise molecular aspects of

TCR-A^k/p46-61 interaction in B10.A(4R) and CBA/J (or B10.A) mice in view of the above two alternative possibilities (b and c) are currently in progress.

Several cases are known where an antigenic determinant can be presented and the appropriate TCRs are present, but no T cell response ensues (19, 50–53). This immune "ignorance" should be distinguished from "true" self-tolerance where the T cells are unable to respond, but has been mechanistically difficult to define. Ignorance has been used in referring to the failure of T cell interaction with the MHC–Ag complex, whereas an earlier stage, the lack of access of certain antigenic determinants to MHC molecules, fits under the term "crypticity" (54); in many senses, these failures of interaction are parallel. Several candidate mechanisms can be suggested: (a) Actually, the T cells are present but are not activated owing to a failure in costimulation necessary for their activation. (b) The T cells are antagonized by related peptides in the local milieu. (c) Suppressive elements, either cells or molecules, exert a negative influence in the local milieu, which however may be circumvented elsewhere. (d) Lack of access of the T cells to their stimulus occurs either because of (i) sequestration of the antigen or T cells, (ii) processing of antigen occurs but not its presentation because of insufficient MHC molecules available, or (iii) steric hindrance: a physical obstruction preventing optimum interaction of the T cell with its ligand. In this work, evidence has been gathered favoring mechanism (d–iii).

It is now well-established that like foreign antigens, self-antigens are also processed and presented by APCs (21, 55–57). Furthermore, the display of self-determinants in the thymus is a critical factor in bringing about both negative and positive selection (1, 4, 8, 58–61). Previous studies from our laboratory and others have shown that T cells po-

tentially directed against cryptic self-determinants escape tolerance induction (21, 62–64). We predict that there will be several determinants from a variety of self-proteins that are processed efficiently by thymic APC and bind well to MHC molecules, but are not presented efficiently to developing T cells due to the presence of hindering epitopic residues. In this situation, in the negatively selecting milieu, the interaction between the TCR and MHC/peptide complex would be very inefficient. Consequently, T cells potentially directed against such self-determinants would escape tolerance induction, but perhaps become involved in positive selection (58) leading to enrichment of the T cell repertoire. The component of the T cell repertoire described above would not pose a threat to the individual under normal circumstances. However, under conditions leading to aberrant or upregulated antigen processing (65, 66), a particular determinant bearing a hindering residue may be processed differently so that the hindering residue is removed, resulting in efficient presentation of the immunogenic self determinant to ambient, autoreactive T cells leading to induction of autoimmunity. Often, as in the case of the CBA/J or the B10.A mouse, but not in the B10.A(4R), a self-peptide in conjunction with self-MHC will positively select a TCR repertoire member that is unhindered by R61 on ML p46-61: in such a case, a response will ensue. Further in regard to autoimmunity, the susceptibility of the members of a heterogeneous population to an autoimmune disease is generally evaluated in the context of the MHC haplotype of the individuals (67, 68). However, our results point to the fact that although the MHC might be capable of binding and presenting a particular pathogenic autoantigenic determinant, the composition of an individual's peripheral TCR repertoire can clearly influence susceptibility to autoimmunity.

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