

# Dominant Determinants in Hen Eggwhite Lysozyme Correspond to the Cryptic Determinants within Its Self-homologue, Mouse Lysozyme: Implications in Shaping of the T Cell Repertoire and Autoimmunity

By Kamal D. Moudgil and Eli E. Sercarz

---

*From the Department of Microbiology and Molecular Genetics, Life Sciences Building, University of California at Los Angeles, Los Angeles, California 90024-1489*

## Summary

We have studied the mouse lysozyme (ML) peptide-specific T cell repertoire in mice of five different major histocompatibility complex (MHC) class II haplotypes. 14 ML peptides were tested in a lymph node T cell proliferation assay. Upon immunization of diverse mouse strains with native ML, there was no response to any of the ML peptides tested. However, nine peptides were immunogenic, although there was no consistent pattern of reactivity toward any peptide among these strains. Thus, an autoreactive T cell repertoire directed against cryptic self(ML)-determinants exists, and it is different in mice of different MHC haplotypes. Moreover, our results demonstrate that crypticity is MHC associated and not merely a structural attribute of the determinant. On comparison of the pattern of response of various peptides of ML and that of its foreign homologue, hen eggwhite lysozyme (HEL) in H-2<sup>k</sup>, H-2<sup>b</sup>, and H-2<sup>d</sup> strains of mice, a striking correlation was evident. The stretches of amino acid sequences of determinants within HEL that were dominant in each of these three strains, almost exactly overlapped in position with those of the cryptic ML determinants against which self-reactivity was demonstrated in the same strain. These results demonstrate that the dominance-crypticity relationship between HEL and ML resulting from differential processing of these two proteins is critical in determining the response to HEL rather than the degree of sequence difference between them. These observations have important implications in the shaping of the T cell repertoire for foreign proteins and in the pathogenesis of autoimmunity.

The repertoire of mature  $\alpha/\beta$  T cells found in the blood and peripheral lymphoid organs is selected by MHC products and self-antigens expressed in the thymus. Immature thymocytes that express TCR with a threshold affinity for self-MHC-self-peptide complexes preferentially emigrate from the thymus into the periphery (positive selection), whereas autoreactive thymocytes that recognize self-antigen-MHC complexes with a high affinity are deleted (negative selection). Self-tolerance is established in part through negative selection, and in part through peripheral mechanisms such as anergy induction, downregulation of TCR, or suppression (1-10).

Lorenz and Allen (11) provided functional evidence in mice that APC from several tissues can process and present the self-antigen, hemoglobin (Hb).<sup>1</sup> They demonstrated that

these APC could stimulate self-Hb-specific hybridomas without addition of exogenous antigen, indicating that determinants within self-Hb are expressed on the surface of the APC. Stockinger and Lin (12) have described that intracellular pro-C5 (the precursor of the fifth component of complement) was processed and presented by macrophages from C5-deficient mice, and could activate class II-restricted C5-specific T cell clones. We have previously reported that two self-class II peptides (A<sub>k</sub> 1-18 and A<sub>b</sub> 1-16) and one self-class I peptide (L<sup>d</sup> 61-85) were immunogenic in B10.A mice, but another self-class I peptide (D<sup>d</sup> 61-85) was nonimmunogenic although it could bind with high affinity to the A<sup>k</sup> molecule and presumably induced tolerance during development (13). Similar results have also been reported using peptides containing sequences from the  $\alpha$  and  $\beta$  chains of the I-A<sup>d</sup> molecule (14). Thus, there are two functional sets of self-determinants, those that are easily processed and presented, readily tolerizing developing T cells and comprising the dominant self, and those that are difficult to present and do not tolerize, comprising the cryptic self.

At present, it is believed that if a determinant within a

---

<sup>1</sup> Abbreviations used in this paper: t-Boc, tert-butyloxycarbonyl; DIC, *N,N*-diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DCM, dichloromethane; Hb, hemoglobin; HEL, hen eggwhite lysozyme; hsp, heat-shock protein; ML, mouse lysozyme.

foreign protein is distinct from the corresponding determinant on the homologous self-protein, then its chances of being immunogenic are high. However, we suggest that the relationship between crypticity and dominance in the host antigen vis-à-vis its homologue, rather than the degree of sequence difference/foreignness between the two proteins, is the major factor in determining the immune response to a foreign protein. Immunogenicity of related foreign determinants can be entirely dependent on the level of crypticity of the determinants in the self-homologue. High crypticity will ensure that T cells directed against the self-determinants are not purged from the repertoire. Moreover, as a result of differential processing of the self and foreign proteins owing to differences in amino acid sequences flanking each of the potential epitopes, determinants that have an identical position in the two proteins might nevertheless show a different pattern (dominant or cryptic) of immunogenicity.

We have now examined this issue using mouse lysozyme (ML) as a model self-protein and the results described in this study support the aforementioned hypothesis. Different cryptic determinants within ML were found in mice of five different MHC haplotypes, each strain revealing a unique pattern of crypticity. Moreover, the determinants on hen eggwhite lysozyme (HEL), a foreign homologue of ML, that correspond in position to the cryptic ML determinants were dominant. These results suggest that crypticity of self(ML)-determinants plays a critical role in influencing the immunogenicity of determinants on HEL.

## Materials and Methods

**Mice.** BALB/c (H-2<sup>d</sup>), B10.D2 (H-2<sup>d</sup>), CBA/J (H-2<sup>k</sup>), B10.PL (H-2<sup>n</sup>), BALB.B (H-2<sup>b</sup>), C57BL/6 (=B6) (H-2<sup>b</sup>), and SJL (H-2<sup>t</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility.

**Peptide Synthesis and Purification.** ML peptides were synthesized by three different methods: (a) ML peptides (mostly 15-mers) were 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 (15). Peptides were generated on *tert*-butyloxycarbonyl (t-Boc) amino acid-Pam resins contained within porous polypropylene bags. These resins and t-Boc amino acid residues having the appropriate side chain protecting groups were obtained from Advanced ChemTech, Louisville, KY, and Peninsula Laboratories, Inc., Belmont, CA. A typical cycle of reactions included deblocking (or deprotection), neutralization, coupling, and washing. The coupling steps were done individually but other steps were done with all teabags in a common solution. Deblocking was done with 55% TFA in dichloromethane (DCM) containing 2-methylindole (0.05%, final concentration). Following deblocking, the resins were washed sequentially in DCM, 2-propanol, DCM, 5% *N,N*-diisopropylethylamine (DIEA) in DCM, and DCM. During the interim period, the appropriate t-Boc amino acid residues were activated either with *N,N'*-diisopropylcarbodiimide (DIC)/DCM (for DIC esters) or with DIC/*N,N*-dimethyl formamide (DMF) (for 1-hydroxybenzotriazole esters). Upon completion of postdeblocking washes, the activated amino acids were added to the resins to allow coupling of the next amino acid in the sequence. The coupling reaction was allowed to proceed for at least 6 h or overnight. After

the coupling reaction was over, the resins were washed sequentially in DCM, DMF, and DCM. Thereafter, the efficiency of coupling was tested on a small sample of the resin by the ninhydrin test. If needed, the resin was subjected to recoupling. After the last coupling of each peptide, a final deblocking was done. The reagents and solvents used in the above-mentioned cycle of reactions were obtained from Aldrich Chemical Company, Milwaukee, WI; Fisher Scientific, Fair Lawn, NJ; and Sigma Chemical Company, St. Louis, MO.

After the final deblocking, peptides were cleaved from the resin using the low trifluoromethane sulphonic acid cleavage method (Applied Biosystems, Inc., Foster City, CA). The peptides were then desalted by passing over a Sephadex G10 column (Pharmacia Fine Chemicals, Piscataway, NJ) using 30% acetic acid. The peptide fraction was analyzed by HPLC (Gilson Medical Electronics, Inc., San Diego, CA) using a Hi-Pore C-18 column (Bio-Rad Laboratories, Richmond, CA), and was further purified as a single peak on a preparative column (G4; Brownlee Labs., Santa Clara, CA) with a solvent system of 0.1% TFA and an increasing gradient of acetonitrile. All purified peptides were subjected to amino acid composition analysis. For this purpose, the peptide sample was hydrolyzed in 6 N HCl at 110°C for 18 h under vacuum in a nitrogen atmosphere, and the Waters Pico-Tag method (Millipore, Milford, MA) was used for determining the amino acid composition of the peptides (16). (b) A complete series of overlapping peptides spanning the entire sequence of ML were synthesized by Dr. H. M. Geysen (Chiron Mimotopes, Clayton, Australia) using the "multipin" peptide synthesis technique. The procedure had been modified as described in detail elsewhere so that the peptides could be cleaved from the pins (17). The first amino acid residue added in each case was proline followed by Boc-lysine(Fmoc)-OH. The Fmoc protecting group was then removed, and additional Fmoc-protected amino acids were added onto the  $\epsilon$ -amino group of the lysine by repetitive cycles of Fmoc deprotection and amino acid couplings. The terminal amino group of each peptide was acetylated. After removal of all the protecting groups, cleavage from the pins was performed by exposure to neutral pH, under which conditions the COOH-terminal lysine-proline residues formed diketopiperazine. Peptide yield was estimated as described (17). (c) Some of the ML peptides were synthesized by Dr. Craig Miles, Macromolecular Resources, Colorado State University, Fort Collins, CO. Manual or automated peptide synthesis was performed using t-Boc amino acids. The amino acid residues were activated with 0.9 equivalents of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and 1.4 equivalents of DIEA. The peptide resin complex was deprotected with 50% TFA, washed, and neutralized with DIEA. The activated amino acids were stirred into the peptide-resin complex and reacted for 30 min. The coupling efficiencies were determined by the ninhydrin assay. The peptides were removed from the synthesis resin in 90% hydrogen fluoride with 10% ethanedithiol as a cation scavenger. The crude products were purified using HPLC.

**Preparation of Mouse Lysozyme (ML).** ML is secreted by J774A.1 cells (American Type Culture Collection, Rockville, MD), a mouse-monocyte-macrophage cell line that synthesizes ML. This cell line was maintained in DMEM (Gibco Laboratories, Grand Island, NY) containing 10% heat-inactivated FCS (Gemini Bioproducts, Inc., Calabasas, CA),  $5 \times 10^{-5}$  M 2-ME (Sigma Chemical Co.), 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (all from Gibco Laboratories, Inc.). Then it was grown as a tumor in the peritoneal cavity of BALB/c mice, which were injected intraperitoneally with 0.5 ml of 2,6,10,14-tetramethyl pentadecane (Pristane; Aldrich Chemical Co., Inc.) 1 wk before injecting  $1-2 \times$

10<sup>7</sup> J774A.1 cells. After 2–3 wk, the ascitic fluid was tapped from the peritoneal cavity, centrifuged at 1,500 rpm for 10 min, dialyzed against PBS, and then used as a source of ML. The activity of ML was tested using the *Micrococcus lysodeikticus* lysis assay (18). The concentration of ML in the ascitic fluid was as high as 300 µg/ml.

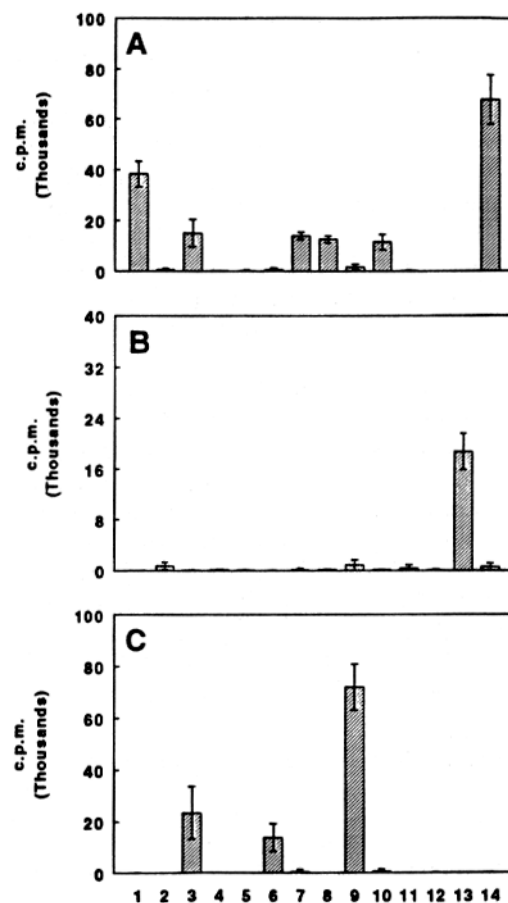
**Lymph Node Proliferation Assay.** Groups of at least four to six mice (8–16 wk old) were immunized with 7 or 14 nmol per mouse of a ML peptide or with 14 or 21 nmol per mouse of native ML in saline or PBS, in a 1:1 (vol/vol) emulsion with CFA containing 1 mg/ml *Mycobacterium tuberculosis* strain H37Ra (Difco Laboratories, Detroit, MI), in the hind footpads. After 9 d, the popliteal lymph nodes were removed and cell suspensions prepared. The debris was allowed to settle, and the cells in the supernatant were washed twice with HBSS (Gibco Laboratories Inc.). The lymph node cells were cultured in 96-well plates at 5 × 10<sup>5</sup> cells/well, in duplicate or triplicate samples, in HL-1 serum-free medium (Ventrex Laboratories, Inc., Portland, ME) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, and different concentrations (1.75–14 µM, final concentration) of the antigen. For the pin peptides, one or two wells were tested per peptide. Tuberculin purified protein derivative (Evans Medical Limited, Horsham, England) was used at a final concentration of 2,000 U/well as a positive control. 1 µCi of [<sup>3</sup>H]thymidine (International Chemical and Nuclear, Irvine, CA) was added per well for the last 18 h of a 5-d culture. Then, the cells were harvested on Printed Filtermat A glass fiber filter (Wallac, Turku, Finland) using a Micro Cell Harvester (Skatron Instruments, Inc., Sterling, VA) and the incorporation of radioactivity was assayed by liquid scintillation counting, using a Betaplate counter (1205; LKB Instruments, Inc., Gaithersburg, MD). The results from a single animal were recorded as mean cpm of duplicate or triplicate cultures, with background (medium alone) values subtracted. The final results from a group of animals immunized with the same peptide were expressed as mean cpm ± SD. The results obtained with ML peptides synthesized by the “teabag” method were confirmed with pin peptides as well as with peptides obtained from Macromolecular Resources, and vice versa.

ML	HEL
1-15 K V Y E R C E F A R T L K R N - - F G - - - L - A A M - - H	
10-23 R T L K R N G H A G Y Y G V A A M - - H - L D M - R - Y	
19-33 G Y Y G V S L A D W V C L A Q N - R - Y - - G N - - - A - A - K	
22-36 G V S L A D W V C L A Q H E S - Y - - G N - - - A - K F - -	
31-45 L A Q H E S N Y N T R A T N Y A - K F - - - F - - Q - - - R	
34-47a H E S N Y N T R A T N Y N R G F - - - F - - Q - - - R - T *	
37-50 N Y N T R A T N Y N R G D Q S - F - - Q - - - R - T * - G -	
40-53 T R A T N Y N R G D Q S T D Y - Q - - - R - T * - G - - - -	
66-79 D G K T P R A V N A C G I N - - R - - G S R - L - N - P	
77-91 G I N C S A L L Q D D I T A A N - P - - - - - S S - - - - S	
95-109 A K R V V R D P Q G I R A W V - - K I - S - G N - M N - - -	
99-113 V R D P Q G I R A W V A W R A - S - G N - M N - - - - - H	
105-119 I R A W V A W R A N C Q N R D M N - - - - - N R - K G T -	
116-129 Q N R D L S Q Y I R N C G V K G T - V Q A W - - G - R L	

**Figure 1.** Amino acid sequences of 14 ML peptides used in this study. For each peptide, the sequence of ML peptide is given on the top, and that for the corresponding HEL peptide immediately below. Only those amino acid residues of the HEL peptide that differ from the amino acid residue of ML at the particular position are depicted. The single-letter amino acid code is used. The asterisk at position 48 in HEL corresponds to an extra amino acid (G) in ML at that position. For accurate comparison of the boundaries and sequences of peptides of ML and HEL, we have numbered G48 in ML as “G47a.” The lyszyme sequences were obtained from the National Biomedical Research Foundation data base.

## Results and Discussion

**Mice of Different MHC Haplotypes Have Unique Patterns of Response to Cryptic ML Determinants.** 14 ML peptides (Fig. 1) were tested in lymph node proliferation assay in mice of three different MHC haplotypes: BALB/c (H-2<sup>d</sup>), CBA/J (H-2<sup>k</sup>), and C57BL/6 (H-2<sup>b</sup>). From this set of partially overlapping ML peptides, whose boundaries were sometimes chosen to avoid difficult synthetic situations, five were nonimmunogenic in each of the three strains. Of the remaining nine peptides, BALB/c mice gave a positive response to only one peptide, whereas CBA/J and C57BL/6 mice responded to seven and three peptides, respectively (Fig. 2). The results obtained with ML peptides synthesized by the “teabag” method were confirmed with pin peptides as well as with



**Figure 2.** The in vitro T cell proliferative response of LN cells to ML peptides in mice of different MHC haplotypes. Mice were immunized in the hind footpads with one of the ML peptides emulsified in CFA, and after 9 d, the response of the LN cells was recalled in vitro with the same peptide. A, B, and C correspond to CBA/J (H-2<sup>k</sup>), BALB/c (H-2<sup>d</sup>), and C57BL/6 (H-2<sup>b</sup>) mice, respectively. The peptide number shown corresponds to the amino acid residues of ML as follows: 1, 1–15; 2, 10–23; 3, 19–33; 4, 22–36; 5, 31–45; 6, 34–47a; 7, 37–50; 8, 40–53; 9, 66–79; 10, 77–91; 11, 95–109; 12, 99–113; 13, 105–119; and 14, 116–129. The response of ML peptides (mean cpm ± SD) shown represents results from four to six animals of each strain. The response to purified protein derivative for each peptide is not shown, but it ranged from 22,769 to >200,000 cpm in different experiments.

peptides obtained from Macromolecular Resources, and vice versa (data not shown). Thus, each of the three strains of mice tested had ML-peptide reactive T cells in their repertoire. Importantly, each strain revealed a unique pattern of response to the panel of ML peptides. These results are shown in tabular form in Table 1, along with partial results from two other independent MHC haplotypes, H-2<sup>u</sup> (B10.PL) and H-2<sup>s</sup> (SJL). Again, the pattern of reactivity to ML peptides appears unique to each haplotype. The data in Table 1 demonstrate that: (a) BALB/c mice respond to only one ML peptide, peptide 105–119 (p105–119), whereas SJL (and also CBA/J, B10.PL, or C57BL/6) mice do not respond to that peptide. However, SJL mice do give a consistent weak response to two other ML peptides (p66–79 and p77–91); (b) CBA/J but not C57BL/6 mice respond to peptides 1–15, 37–50, 40–53, and 116–129, whereas C57BL/6 but not CBA/J mice respond to peptide 34–47a. However, both CBA/J and C57BL/6 mice respond to peptides 19–33 and 66–79, although the degree of response to peptide 66–79 is quite different in the two strains; (c) both B10.PL and CBA/J mice respond to peptide 116–129, whereas BALB/c, C57BL/6, or SJL mice fail to give a response to this peptide; however, CBA/J and B10.PL mice differ in their response to peptide 19–33; (d) although both CBA/J and SJL mice give a consistent weak response to peptides 66–79 and 77–91, only CBA/J mice respond to peptide 116–129. In brief summary, collective responses to ML were scattered throughout the molecule with each strain showing a reproducible and limited pattern of T cell proliferative response.

Upon immunization of mice with the native ML emulsified in CFA, there was no recall response to any of the ML peptides tested (data not shown). Since nine of these ML peptides were immunogenic when injected in the peptide form (Table 1), these results suggest that the determinants corresponding to the immunogenic ML peptides are either not generated from the processing of the whole ML molecule, or they are generated but not efficiently presented to T cells to induce a response. In this context, the immunogenic ML peptides represent “cryptic” self-determinants. ML is a self-protein that is present in the blood, and the protein as well

as its messenger RNA have been shown to be also present in the thymus and other tissues (19, 20). During development of the T cell repertoire, dominant determinants within ML should lead to deletion or anergy of the ML-reactive T cells. On the contrary, T cells potentially reactive to cryptic ML determinants would evade tolerance induction, permitting these T cells to be recruited in adulthood by specific peptides to induce a response (21–23). Thus, we provide evidence that self-reactivity exists to several cryptic ML determinants in mice, and that the ML-specific autoreactive T cell repertoire is different in mice of different MHC haplotypes.

*Crypticity Is MHC Associated.* To determine the influence of MHC and non-MHC (background) genes on the pattern of crypticity of determinants within ML, the response of six ML peptides was tested in mice of three different MHC haplotypes, including MHC-congenic strains, using the lymph node proliferation assay. The results are given in Table 2. It was found that the pattern of response of different ML peptides in C57BL/6 (H-2<sup>b</sup>) mice was completely different from that in B10.D2 (H-2<sup>d</sup>) or B10.PL (H-2<sup>u</sup>) mice of disparate MHC, whose background is almost identical to that of C57BL/6 mice. On the contrary, the pattern of response of C57BL/6 mice to the same peptides correlated precisely to that of BALB.B (H-2<sup>b</sup>) mice, which have the same MHC as C57BL/6 mice but a different non-MHC background. Similarly, the pattern of response of BALB/c (H-2<sup>d</sup>) mice was identical to that of B10.D2 (H-2<sup>d</sup>) mice of the same MHC haplotype, but with different non-MHC genes. On the contrary, there was no similarity, whatsoever, in the patterns of response of BALB/c (H-2<sup>d</sup>) and BALB.B (H-2<sup>b</sup>) mice, which have the same background but are of different MHC haplotype. These observations clearly demonstrate that a cryptic determinant in one haplotype is not necessarily cryptic in another: crypticity is MHC associated and not merely a structural attribute of the determinant.

The strong association between the crypticity of a determinant and the MHC of the host could be attributed to the ability of a potentially cryptic determinant to bind to MHC class II molecules of a particular haplotype, as well as to the predominant influence of MHC genes on the development

**Table 1.** *The Pattern of Response of ML Peptides in Mice of Different MHC Haplotypes*

Strain	ML Peptide Tested													
	1–15	10–23	19–33	22–36	31–45	34–47a	37–50	40–53	66–79	77–91	95–109	99–113	105–119	116–129
BALB/c	–	–	–	–	–	–	–	–	–	–	–	–	+	–
CBA/J	3+	–	3+	–	–	–	+	+	±	±	–	–	–	4+
C57BL/6	–	–	3+	–	–	2+	–	–	3+	–	–	–	–	–
SJL	ND	–	ND	ND	ND	ND	ND	ND	±	±	–	–	–	–
B10.PL	ND	–	–	–	ND	ND	ND	ND	–	–	–	–	–	3+

Mice of different MHC haplotypes were immunized in the hind footpads with one of the ML peptides emulsified in CFA. After 9 d, lymph node cells were tested for a proliferative response to the same peptide *in vitro*. The results of the assay were recorded as a stimulation index (S.I.) = cpm with antigen/cpm without antigen. The response to each peptide was determined in four to six individual mice. Based on the value of average S.I., the response to each peptide was arbitrarily graded as follows: S.I. of <3, –; 3–3.9, ±; 4–9.9, +; 10–24.9, 2+; 25–49.9, 3+; and 50–100, 4+.

**Table 2.** Profiles of Responses to ML Peptides in MHC-congenic Strains of Mice

ML peptide	B6 (H-2 <sup>b</sup> )	BALB.B (H-2 <sup>b</sup> )	BALB/c (H-2 <sup>d</sup> )	B10.D2 (H-2 <sup>d</sup> )	B10.PL (H-2 <sup>a</sup> )
19-33	3+ (48.7)	3+ (30.8)	- (1.4)	- (1)	- (1.6)
22-36	- (1)	- (1.6)	- (2.3)	- (1.5)	- (1.6)
66-79	3+ (43.7)	2+ (16.4)	- (1.4)	- (1.3)	- (2.1)
95-109	- (1)	- (1.6)	- (1)	- (1.6)	- (2)
105-119	- (1)	- (1.9)	3+ (32.9)	3+ (44.9)	- (2.1)
116-129	- (2.1)	- (1.7)	- (1)	- (1.2)	3+ (48.8)

Mice of three different MHC haplotypes, including MHC congenic strains, were immunized in the hind foot pads with one of the ML peptides emulsified in CFA, and after 9 d, the lymph node cell proliferation assay was performed. The response to each peptide, determined in four individual mice, was graded as described in Table 1. The figures in parentheses represent average stimulation indices.

of the T cell repertoire. In a recent study (Grewal, I. S., and E. E. Sercarz, manuscript in preparation), it was found that non-MHC genes had a limited effect on the expression of the T cell repertoire. In mice of six different non-MHC backgrounds but identical MHC (A<sup>b</sup>), there was only one anti-HEL repertoire difference among all these strains. However, in another study (Grewal, I. S., K. D. Moudgil, and E. E. Sercarz, manuscript submitted for publication), we have observed that a determinant within HEL that induces a strong T cell response in one strain of mice can be nonimmunogenic in another strain of mice of identical MHC but of different background. The nonresponder strain was found to be defective in its ability to "fine-process" that determinant, which was critical for its immunogenicity. Therefore, the influence of non-MHC genes on crypticity of a determinant cannot be completely ruled out.

*Comparison of the Patterns of the T Cell Response of H-2<sup>k</sup>, H-2<sup>b</sup>, and H-2<sup>d</sup> Strains of Mice to ML and HEL.* In view of the above observations, it was pertinent to ask if there was any relationship between self-reactivity to ML peptides and the T cell response to determinants on the homologous foreign antigen, HEL. The T cell response to HEL has been extensively studied in our laboratory (21, 24-27) and by others (28, 29). Upon immunization with HEL (emulsified in CFA) in the footpad, CBA/J (or B10.A/C3H), C57BL/6, and BALB/c mice reveal characteristic profiles of lymph node T cell proliferative responses when tested with various HEL pep-

**Table 3.** Relationship between the Cryptic Determinants within ML and Dominant Determinants on HEL in Mice of Different MHC Haplotypes

ML	H-2 <sup>k</sup>		H-2 <sup>b</sup>		H-2 <sup>d</sup>	
	HEL	ML	HEL	ML	HEL	ML
1-15	1-17*	-	-	-	-	-
19-33	18-33†	19-33	20-35	-	-	-
37-50	34-45	34-47a	30-53	-	-	-
40-53	ND	-	-	-	-	-
ND	46-61	ND	-	ND	-	-
66-79	74-86§	66-79	74-90§	-	-	-
77-91	74-86§	-	74-90§	-	-	-
-	-	-	-	105-119	106-116	-
116-129	116-129	-	-	-	-	-

\* Codominant in C3H and CBA/J, but cryptic in B10.A mice.

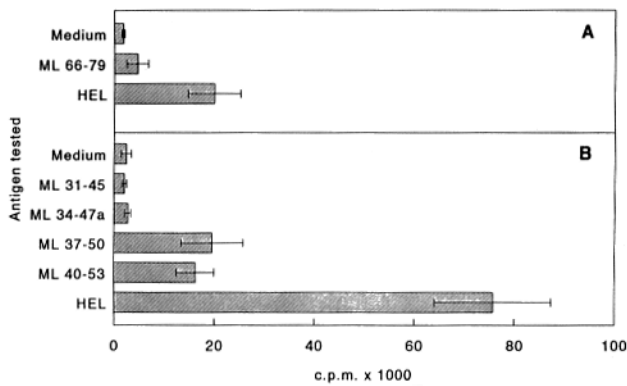
† HEL peptide 20-35 also gives an equally good response.

§ 74-86 is a subdominant determinant in mice of H-2<sup>k</sup> haplotype. At this time, we do not know the minimal determinant that is recognized by H-2<sup>k</sup> or H-2<sup>b</sup> strains in ML 66-79. Therefore, the corresponding HEL determinant is repeated for 74-86 (H-2<sup>k</sup>) as well as 74-90 (H-2<sup>b</sup>).

The peptides listed under ML are the ones that are immunogenic, cryptic peptides in mice of that particular MHC haplotype. The peptides depicted under HEL represent dominant determinants within HEL, to which mice of that MHC haplotype respond after immunization with native HEL. Statistical analysis of the data was done by computing the *p* value, to evaluate the statistical significance of the observed concordance of strain-specific cryptic and dominant determinants. The *p* value was found to be 0.082 for H-2<sup>k</sup> mice, 0.022 for H-2<sup>b</sup> mice, and 0.049 for H-2<sup>d</sup> mice. All these three values of *p* were highly significant.

tides (Table 3). Also listed in Table 3 are the cryptic ML peptides that were found to be immunogenic in mice of three different MHC haplotypes.

In each of the three strains of mice of different MHC haplotypes tested, there is a remarkable correlation between the lymph node T cell proliferative responses to ML peptides and HEL. In particular, the positions of the determinants within ML that are cryptic in H-2<sup>k</sup> mice match almost precisely the dominant determinants on HEL that induce a response in that strain when the native molecule is used as immunogen. Surprisingly, some of these ML peptides differ from the corresponding HEL peptides by as many as 7-10 amino acids (Fig. 1). A similar correlation is observed in mice of the H-2<sup>b</sup> and H-2<sup>d</sup> haplotypes. The only "mismatches" are in the area from 66-86. These results demonstrate that ML and HEL are processed differentially: regions dominant in HEL and presumably readily processed, are cryptic on ML, but immunogenic when presented as ML peptides. It has been shown that even a single amino acid substitution can alter T cell determinant selection during antigen processing (30). Accordingly, differences in the processing of ML and HEL can be attributed to the vast differences between the amino acid sequences of these two related proteins (55 amino acid residues/130 amino acids). Thus, sufficient variation is provided



**Figure 3.** Priming of T cells by cryptic ML determinants for response to HEL. CBA/J mice were immunized in a hind footpad with ML peptide 66–79 (A) or with a mixture of ML pin peptides 31–45, 34–47a, 37–50, and 40–53 (B) emulsified in CFA. After 9 d, the response of the LNC was recalled with the ML peptide used for immunization or with HEL. In B, the response was recalled separately with each component of the mixture of peptides used for immunization. In each panel, the response of four animals is shown as mean cpm  $\pm$  SD. Medium, medium control. The response of mice to PPD was 104,092 (A), and 143,464 (B).

in the flanking regions of each potential determinant to permit a diversity of new processing sites.

What is the relationship among the antigenic determinants on protein antigens homologous to a self-protein (a frequent situation in immunology)? Among the possible determinants on the related molecule, which ones will be immunogenic for T cells? The T cell repertoire to a foreign antigen such as HEL might have been predicted to be related to determinants that are distinct from self, e.g., determinants with unique agretopes or epitopes to which the animal has not been tolerized. In fact, there are few very similar regions between ML and HEL; thus, the nearly precise overlap of cryptic ML and dominant HEL determinants excludes the importance of difference per se. We noticed in the H-2<sup>k</sup> B10.A, C3H, and CBA/J strains that there are many codominant determinants on HEL that are able to activate the available T cells, while in the B10.D2 or BALB/c H-2<sup>d</sup> strains, only one determinant is dominant (21, 24–29). In the case of H-2<sup>d</sup> BALB/c mice in particular, one might have predicted a response to other cryptic determinants on ML or other dominant determinants on HEL than 106–116. However, no consistent response has been seen to other HEL determinants in this strain of mouse, even though certain HEL peptides are known to bind to the A<sup>d</sup> (e.g., 11–25, 20–35, 74–86) or the E<sup>d</sup> molecule (e.g., 1–17) (31; Apple, R. J., H.-K. Deng, A. Miller, E. E. Sercarz, and J. Cogswell, manuscript in preparation). Therefore, the lack of response of BALB/c mice to determinants on HEL other than 106–116 is clearly not due to inability of those determinants to bind to the MHC molecules. Furthermore, based on the above information on MHC binding, we have excluded the possibility that the identical patterns of response to determinants within ML and HEL were due to limited agretypy, i.e., only certain regions in HEL and the corresponding regions in ML had the ability to bind to MHC molecules in the BALB/c mice. In any case, the above-

mentioned possibility would have neither changed the most important conclusion of this study that the ML determinants corresponding to dominant HEL determinants were cryptic, nor would it have undermined the importance of the crypticity of certain self(ML)-determinants in allowing potentially self-reactive T cells to escape tolerance induction.

It may be concluded that differences in the patterns of response to HEL in mice of different MHC haplotypes represent a conjunction of certain features: (a) the high-affinity binding of the favored determinants on HEL that are dominant and easily processed, and (b) the existence of a T cell repertoire that has not been rendered tolerant by the related ML determinants. It is noteworthy that the repertoire is available because the homologous determinants on the self-protein are cryptic. (In fact, this category of determinants may also include apparently dominant self-determinants that for some reason have not rendered the potentially self-reactive T cells tolerant, for example, the Ac1–9 determinant on myelin basic protein may have been inefficiently processed or presented by the animal's APC.) Accordingly, the unusual relationship we have described is explicable on the basis of a fortuitous congruence of crypticity and dominance of determinants within ML and HEL in each of these strains.

*Priming of HEL Cross-reactive T Cells by Cryptic ML Self-peptides.* A prediction that emerges from this congruence of position is that cross-reactivity should exist between the cryptic self-ML peptides and dominant determinants on the “foreign” congener, HEL, despite the 55-amino acid difference between the two proteins. In a preliminary set of experiments to explore the above prediction, CBA/J mice were immunized in a hind footpad with ML peptide 66–79 or with a mixture of pin peptides (31–45, 34–47a, 37–50, and 40–53 amino acid residues of ML), and the lymph node response was recalled in vitro with HEL, or with each of the single peptides employed in the initial immunization. The results are given in Fig. 3. These results demonstrate that the cryptic ML peptides successfully primed T cells, which were then recruited during the in vitro recall response to HEL. Interestingly, the level of proliferation elicited to HEL was heteroclitic and far superior to that induced by the peptide itself. This may relate to the findings by others showing the importance of B cells in the T cell response to self-antigens (22). Possibly, the great disparity between response to the peptide and HEL favors the idea that B cell priming to 66–79 or other ML peptides mentioned above allowed B cells cross-reactive for HEL to then present other determinants on HEL to ambient T cells in vitro. Such activation of naive T cells has not previously been examined in this system and experiments to test the validity of this explanation are in progress.

We can postulate that frequently during the lifetime of the animal, e.g., after subclinical bouts of infection, ML will be produced in excess owing to defensive reactions and up-regulation of antigen presentation should follow (32). Certain cryptic ML determinants, those that would be the relatively most easily processed and displayed, will provide a covert expansion signal to specific T cells. Particular highly cross-reactive members within this expanded memory T cell reper-

toire would then be selected by relatively dominant determinants on HEL after antigen injection. It may only be a small subset of the T cells directed against a cryptic self ML determinant that would be sufficiently cross-reactive with HEL. We suggest that T cells with the potential to react with particular cryptic self(ML)-determinants are the ones that are stimulated by related determinants on the homologous protein, HEL, although marked differences can be present in peptide sequence. Recent studies in this laboratory describing the high level of degeneracy in recognition by individual T cell receptors (reference 32a; Deng, H.-K., and E. E. Sercarz, manuscript in preparation; and Nanda, N. K., K. K. Arzoo, H. M. Geysen, A. Sette, and E. E. Sercarz, manuscript submitted for publication) allow us to extrapolate the above results to suggest that the T cell repertoire to a foreign protein is shaped and focused by the cryptic determinants on the homologous self-protein. Detailed studies of ML-HEL determinant cross-reactivity and binding of ML/HEL peptides to MHC molecules to validate this relationship are underway in the laboratory.

Let us extrapolate to an autoimmune condition, arthritis, and explore the involvement of cryptic self-determinants. T cell-mediated responses to a 65-kD heat-shock protein (hsp-60 family) of *M. tuberculosis* have been implicated in the patho-

genesis of inflammatory arthritis both in experimental animals and in humans (33–36). Using synthetic peptides, responses to shared T cell determinants of the mycobacterial and human hsp-60 in patients with rheumatoid arthritis have been reported (35). Based on the ML study reported above, we suggest that shared cross-reactive determinants on mycobacterial hsp-60 in fact are responsible for induction of rheumatoid arthritis, but that a particular constellation in the determinant relationship must exist: the cross-reactive determinants must be dominant on the bacterial hsp to initiate a host response, while they have to be cryptic on the self-hsp. The potentially self-reactive T cells against the cryptic self-determinants are stimulated when the microbial hsp arrives. We predict that exploration of this relationship, which should be true for all cases of “molecular mimicry” (37), would indicate that autoimmunity is inevitably associated with the anti-cryptic T cell repertoire. Furthermore, the immune response to a foreign homologous protein might be susceptible to tolerization with the related cryptic self-determinants and vice versa. This would provide a new therapeutic approach for autoimmune situations, where external pathogens are implicated in initiating the autoimmune process. We are currently investigating these issues.

---

We wish to thank Drs. M. Kronenberg and N. Nanda for critical review of the manuscript, R. Trezza for the “teabag” synthesis and purification of peptides, Dr. A. V. Fowler for performing amino acid composition of the peptides, Dr. J. Baumer for her help in using the National Biomedical Research Foundation data base, Dr. T. S. Ferguson for statistical analysis of the data, and D. Ahmadi for excellent technical assistance.

This work was supported by National Institutes of Health grants AI-11183 and AR-3683406, and American Cancer Society grant IM-626.

Address correspondence to Eli E. Sercarz, Department of Microbiology and Molecular Genetics, 5304 Life Sciences Building, 405 Hilgard Avenue, Los Angeles, CA 90024-1489.

Received for publication 29 April 1993 and in revised form 30 July 1993.

## References

1. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell*. 49:273.
2. Kisielow, P., H. Bluthmann, U.D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4<sup>+</sup>8<sup>+</sup> thymocytes. *Nature (Lond.)*. 333:742.
3. Sha, W.C., C.A. Nelson, R.D. Newberry, D.M. Kranz, J.H. Russell, and D.Y. Loh. 1988. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature (Lond.)*. 336:73.
4. Nikolic-Zugic, J., and M.J. Bevan. 1990. Role of self-peptides in positively selecting the T-cell repertoire. *Nature (Lond.)*. 344:65.
5. Rocha, B., and H. von Boehmer. 1991. Peripheral selection of the T cell repertoire. *Science (Wash. DC)*. 251:1225.
6. Lo, D., J. Freedman, S. Hesse, R.L. Brinster, and L. Sherman. 1991. Peripheral tolerance in transgenic mice: tolerance to class II MHC and non-MHC transgene antigens. *Immunol. Rev*. 122:87.
7. Schonrich, G., U. Kalinke, F. Momburg, M. Malissen, A.-M. Schmitt-Verhulst, B. Malissen, G.J. Hammerling, and B. Arnold. 1991. Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell*. 65:293.
8. Takahama, Y., E.W. Shores, and A. Singer. 1992. Negative selection of precursor thymocytes before their differentiation into CD4<sup>+</sup>CD8<sup>+</sup> cells. *Science (Wash. DC)*. 258:653.
9. Simpson, E., P.J. Dyson, A.M. Knight, P.J. Robinson, J.I. Elliott, and M. Altmann. 1993. T-cell receptor repertoire selection by mouse mammary tumor viruses and MHC molecules. *Immunol. Rev*. 131:93.
10. Fowell, D., and D. Mason. 1993. Evidence that the T cell reper-

- toire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4<sup>+</sup> T cell subset that inhibits this autoimmune potential. *J. Exp. Med.* 177:627.
11. Lorenz, R.G., and P.M. Allen. 1988. Direct evidence for functional self-protein/Ia-molecule complexes in vivo. *Proc. Natl. Acad. Sci. USA.* 85:5220.
  12. Stockinger, B., and R.H. Lin. 1989. An intracellular self protein synthesized in macrophages is presented but fails to induce tolerance. *Int. Immunol.* 1:592.
  13. Benichou, G., P.A. Takizawa, P.T. Ho, C.C. Killion, C.A. Olson, M. McMillan, and E.E. Sercarz. 1990. Immunogenicity and tolerogenicity of self-major histocompatibility complex peptides. *J. Exp. Med.* 172:1341.
  14. Agarwal, B., M. Manickasundari, E. Fraga, and B. Singh. 1991. T cells that recognize peptide sequence of self MHC class II molecules exist in syngeneic mice. *J. Immunol.* 147:383.
  15. Houghten, R.A. 1985. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. USA.* 82:5131.
  16. Cohen, S.A., and D.J. Strydom. 1988. Amino acid analysis utilizing phenylisothiocyanate derivatives. *Anal. Biochem.* 174:1.
  17. Maeji, N.J., A.M. Bray, and H.M. Geysen. 1990. Multi-pin peptide synthesis strategy for T cell determinant analysis. *J. Immunol. Methods.* 134:23.
  18. Parry, R.M., Jr., R.C. Chandan, and K.M. Shahani. 1965. A rapid and sensitive assay of muramidase. *Proc. Soc. Exp. Biol. Med.* 119:384.
  19. Cross, M., I. Mangelsdorf, A. Wedel, and R. Renkawitz. 1988. Mouse lysozyme M gene: isolation, characterization, and expression studies. *Proc. Natl. Acad. Sci. USA.* 85:6232.
  20. Riblet, R.J., and L.A. Herzenberg. 1970. Mouse lysozyme production by a monocytoma: isolation and comparison with other lysozymes. *Science (Wash. DC).* 168:1595.
  21. Gammon, G., and E. Sercarz. 1989. How some T cells escape tolerance induction. *Nature (Lond.).* 342:183.
  22. Lin, R.-H., M.J. Mamula, J.A. Hardin, and C.A. Janeway, Jr. 1991. Induction of autoreactive B cells allows priming of autoreactive T cells. *J. Exp. Med.* 173:1433.
  23. Mamula, M.J. 1993. The inability to process a self-peptide allows autoreactive T cells to escape tolerance. *J. Exp. Med.* 177:567.
  24. Shastri, N., G. Gammon, S. Horvath, A. Miller, and E.E. Sercarz. 1986. The choice between two distinct T cell determinants within a 23-amino acid region of lysozyme depends on their structural context. *J. Immunol.* 137:911.
  25. Gammon, G., J. Klotz, D. Ando, and E.E. Sercarz. 1990. The T cell repertoire to a multideterminant antigen. Clonal heterogeneity of the T cell response, variation between syngeneic individuals, and in vitro selection of T cell specificities. *J. Immunol.* 144:1571.
  26. Gammon, G., N. Shastri, J. Cogswell, S. Wilbur, S. Sadegh-Nasseri, U. Krzych, A. Miller, and E. Sercarz. 1987. The choice of T-cell epitopes utilized on a protein antigen depends on multiple factors distant from, as well as at the determinant site. *Immunol. Rev.* 98:53.
  27. Gammon, G., H.M. Geysen, R.J. Apple, E. Pickett, M. Palmer, A. Ametani, and E.E. Sercarz. 1991. T cell determinant structure: cores and determinant envelopes in three mouse major histocompatibility complex haplotypes. *J. Exp. Med.* 173:609.
  28. Allen, P.M., D.J. McKean, B.N. Beck, J. Sheffield, and L.H. Glimcher. 1985. Direct evidence that a class II molecule and a simple globular protein generate multiple determinants. *J. Exp. Med.* 162:1264.
  29. Adorini, L., E. Appella, G. Doria, and Z.A. Nagy. 1988. Mechanisms influencing the immunodominance of T cell determinants. *J. Exp. Med.* 168:2091.
  30. Liu, Z., K.P. Williams, Y.-H. Chang, and J.A. Smith. 1991. Single amino acid substitution alters T cell determinant selection during antigen processing of *Staphylococcus aureus* nuclease. *J. Immunol.* 146:438.
  31. Sette, A., S. Buus, E. Appella, J.A. Smith, R. Chesnut, C. Miles, S.M. Colon, and H.M. Grey. 1989. Prediction of major histocompatibility complex binding regions of protein antigens by sequence pattern analysis. *Proc. Natl. Acad. Sci. USA.* 86:3296.
  32. Lehmann, P.V., E.E. Sercarz, T. Forsthuber, C.M. Dayan, and G. Gammon. 1993. Determinant spreading and the dynamics of the autoimmune T-cell repertoire. *Immunol. Today.* 14:203.
  - 32a. Bhardwaj, V., V. Kumar, H.M. Geysen, and E.E. Sercarz. 1994. Degerenerate recognition of dissimilar antigenic peptide by myelin basic protein-reactive T cells: implication for thymic education and autoimmunity. *J. Immunol.* In press.
  33. Van Eden, W., J.E.R. Thole, R. van der Zee, A. Noordzij, J.D.A. van Embden, E.J. Hensen, and I.R. Cohen. 1988. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature (Lond.).* 331:171.
  34. Gaston, J.S.H., P.F. Life, P.J. Jenner, M.J. Colston, and P.A. Bacon. 1990. Recognition of a mycobacteria-specific epitope in the 65-kD heat-shock protein by synovial fluid-derived T cell clones. *J. Exp. Med.* 171:831.
  35. Quayle, A.J., K.B. Wilson, S.G. Li, J. Kjeldsen-Kragh, F. Oftung, T. Shinnick, M. Sioud, O. Forre, J.D. Capra, and J.B. Natvig. 1992. Peptide recognition, T cell receptor usage and HLA restriction elements of human heat-shock protein (hsp) 60 and mycobacterial 65-kDa hsp-reactive T cell clones from rheumatoid synovial fluid. *Eur. J. Immunol.* 22:1315.
  36. Henwood, J., J. Loveridge, J.I. Bell, and J.S.H. Gaston. 1993. Restricted T cell receptor expression by human T cell clones specific for mycobacterial 65-kDa heat-shock protein: selective *in vivo* expansion of T cells bearing defined receptors. *Eur. J. Immunol.* 23:1256.
  37. Oldstone, M.B.A. 1989. Molecular mimicry as a mechanism for the cause and as a probe uncovering etiologic agent(s) of autoimmune disease. *Curr. Top. Microbiol. Immunol.* 145:127.